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Type 4 resistant starch diminishes Citrobacter rodentium induced diarrhea in C3H mice

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ABSTRACT

Purpose: The purpose of this study was to examine the effects of a chemically modified resistant starch, RS4, on diarrhea and inflammation induced by *Citrobacter rodentium*. We hypothesized that a diet supplemented with RS4, contributing to 25% resistance of the total starch in the diet (55% starch diet), would significantly improve stool consistency and provide protection against the inflammation associated with the pathogen, including inflammation score, mucosal height, ulceration, goblet cell loss, edema, and hyperplasia.

Design: 36 mice (18 male, and 18 female) were randomly assigned four treatment groups: uninfected mice fed the control starch diet, uninfected mice fed the RS4 supplemented diet, *C. rodentium* infected mice fed the control starch diet, and *C. rodentium* infected mice fed the RS4 supplemented diet. After inoculation with *C. rodentium*, mice were be subjected to the diets for two weeks, and daily food intake, body weight, and stool consistency were measured. At the completion of the two weeks, mice were euthanized and blood was collected via cardiac puncture for serum glucose, insulin, and lipid analysis. Colon and cecum contents were collected and analyzed for pH, stool fat, and water content; and the tissues were sent for histopathology scoring.

Expected results: *C. rodentium* infected mice fed the RS4 supplemented diet were expected to show a significant increase in stool consistency compared to the infected mice the fed the control starch diet. The infected mice fed the RS4 diet were also expected to have a less severe inflammatory response due to the *C. rodentium* compared to the infected mice fed the control diet, which would be seen in the histopathology scores. Body weight loss and decreased food intake due to the *C. rodentium* pathogen were expected to be less severe in mice fed the RS4 supplemented diet, due to its expected protection against inflammation and diarrhea.

CHAPTER I

INTRODUCTION: THESIS ORGANIZATION

This thesis will begin with a review of literature investigating the characterization and properties of starch. The review will then concentrate on resistant starch, its classification and structure. Methods of measuring resistant starch will be mentioned, followed by the effects of resistant starch on the following: blood glucose, insulin, cholesterol, triglycerides, short chain fatty acid production, satiety and weight maintenance, and diarrhea. Adverse effects of resistant starches will be stated, as well as the effects of resistant starch on inflammatory bowel disease. The review will then shift focus to *Citrobacter rodentium*, and its causation of diarrheal illness and inflammation in murine models. The review will finish with a brief overview of the background, methods, hypotheses and expected results of the resistant starch mouse study. Following the review, the materials and methods of the study will be presented. The results of the study will be stated, as well as a discussion of the findings. This thesis will conclude with references and acknowledgements.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Inflammatory bowel disease, including colitis, is a chronic disease without cure. Its trademark symptoms are diarrhea and uncontrollable inflammation of the intestinal mucosa. Resistant starch is thought to be beneficial to gut health, through its fermentation in the large intestine to create short chain fatty acids. The production of short chain fatty acids is thought to improve upon gut barrier function, which can be beneficial to diarrheal and colonic diseases. In conjunction with the production of short chain fatty acids, resistant starch has a high water holding capacity, thought to increase stool bulk and consistency, lessening the degree of water in stool. The purpose of this study was to examine the effects of a type-4 resistant starch (RS4) on diarrhea and inflammation in C3H mice triggered by *Citrobacter rodentium*, an A/E pathogen that causes inflammation and diarrhea similar to that of colitis. It was hypothesized that a diet supplemented with RS4 would primarily increase stool consistency in mice inoculated with *C. rodentium*. The RS4 diet treatment would also cause mice to experience a less severe inflammatory response due to the *C. rodentium*, which include goblet cell loss, mucosal height elevation, hyperplasia, edemas, and ulceration.

Starch Characterization

Starch is one of the main forms of dietary carbohydrates in humans, and contributes to more than 50% of daily energy intake in humans (Topping, Fukushima et al. 2003). In higher plants, starch is synthesized in plastids, and degraded at night to provide substrates for leaf respiration and sucrose synthesis. In tubers, roots and other non-photosynthetic organs, sucrose is converted to starch for long-term storage (Zeeman, Kossmann et al. 2010).

Chemically, starches are polysaccharides with α-1,4 and/or α-1,6 linkages between monosaccharides. Two main structural types of starch exist, amylose and amylopectin. Amylose is a relatively small, linear, and consists of α-1,4 linkages (Bird, Brown et al. 2000, Hoover 2000, Nugent 2005, Englyst, Liu et al. 2007). Due to the linearity of the molecule, amylose is associated with a lessened degree of digestibility compared to amylopectin (Nugent 2005). Amylopectin is a branched molecule with both types of linkages, which contribute to its larger size compared to amylose (Bird, Brown et al. 2000, Nugent 2005). Most commercially available starches have 70-80% amylopectin (Bird, Brown et al. 2000).

Amylose and amylopectin form semi crystalline granules, with two main types. A type is found in cereals and B type is found in tubers and amylose-rich starches. A third crystalline type has been found, C type, and is a mixture of A and B types. C type crystalline structure is primarily found in legumes (Tester, Karkalas et al. 2004, Nugent 2005, Zeeman, Kossmann et al. 2010).

Starches are broken down first through hydrolysis by salivary α-amylases into shorter oligosaccharides. Once the partially digested starch is sent to the gut,

where the pancreatic α-amylases hydrolyze to cleave α-1,4 linkages at random locations. Brush border enzymes convert the products to free glucose. Overall, hydrolysis of starches yields free glucose that can be absorbed and distributed throughout the body (Asp, Van Amelsvoort et al. 1996, Nugent 2005, Lehmann and Robin 2007). Cooking starches with excess water, also known as gelatinization, can increase the degree of hydrolysis (Bird, Brown et al. 2000).

Starch can be divided into different subcategories based upon digestibility, or breakdown by enzymes: rapidly digestible starch, slowly digestible starch, and resistant starch. Rapidly digestible starch, RDS, is found mostly in starches cooked in moist heat. This can include bread or potatoes. RDS is converted to glucose within 20 minutes of enzyme digestion in the small intestine. Slowly digestible starch, SDS, consists of type A and C crystalline structure. SDS is completely digested in the small intestine, but at a slower rate than RDS, and is digested within 20-120 minutes. Resistant starch, RS, has a slowed or no hydrolysis by α –amylase, so that part of the starch reaches the large intestine. The RS value is the difference between what the total starch and the amount of starch hydrolyzed by 120 minutes (Englyst and Hudson 1996, Sajilata, Singhal et al. 2006).

Resistant Starch Classification and Structure

Resistant starch can be defined as any starch that resists digestion in the small intestine, and passes to the large intestine where it is subjected to fermentation (Englyst and Hudson 1996, Nugent 2005). Fermentation of starch in the large intestine yields end products such as hydrogen, carbon dioxide, methane,

and short chain fatty acids (Ferguson, Tasman-Jones et al. 2000, Nugent 2005). The resistance of starch is associated with the interaction between the starch polymers. Due to the lack of branching, amylose is associated with a slower digestion. B and C crystal types are also associated with a lesser degree of digestibility (Nugent 2005).

Resistant starches can be separated into four subtypes based upon degree of physical inaccessibility, granular structure, degree of retrogradation, amylose-amylopectin ratio, and chemical modifications. These subtypes include: RS1, RS2, RS3, and RS4 (Bird, Brown et al. 2000, Nugent 2005, Englyst, Liu et al. 2007).

RS1 starches are physically inaccessible to digestion (Nugent 2005, Sajilata, Singhal et al. 2006). The inaccessibility is due to intact cell walls, which would be seen in grains, seeds and tubers. RS1 starches are heat stable, which is what allows them to be a useful food ingredient. Milling or chewing can help increase digestibility of this type of starch (Bird, Brown et al. 2000, Nugent 2005). Large particles transport more quickly through the gut, with less absorption by the small intestine. Smaller particles would have more absorption in the intestine, with significantly smaller amounts arriving at the large intestine (Topping, Fukushima et al. 2003).

RS2 describes starches in their native granule form. The structure of the granule is the property that protects the starches from digestion (Bird, Brown et al. 2000, Nugent 2005). The granule is tightly packed, leaving it somewhat dehydrated. The compact structures of the granules permit the starch to be partially inaccessible to digestive enzymes (Haralampu 2000, Sajilata, Singhal et al. 2006). A high amylose to amylopectin ratio is present in RS2 starches, and the most commonly used RS2s

are high-amylose starches (Bird, Brown et al. 2000). Foods that contain RS2 include raw potatoes and green bananas. A large benefit to RS2 in the food industry is that it retains its structure and resistance even during food preparation and processing (Nugent 2005).

RS3 is associated with non-granular starches formed during the retrogradation of starch granules. RS3 starches are characterized by their high thermal stability. Retrogradation occurs when starch is cooked past its gelatinization temperature, then cooled. The heating in excess water, or the gelatinization, disrupts the starch granules. Once in the gelatinization phase, the starch is accessible to digestive enzymes. In retrogradation, however, the starches are re-cooled. The cooling period forces the unstable starches to re-crystallize, and the new crystal structures are resistant to amylase hydrolysis (Haralampu 2000, Nugent 2005). Cooked and cooled potatoes are a prime instance of RS3 (Sajilata, Singhal et al. 2006).

RS4 designates chemically or physically modified starches. Chemical modification of RS4 consists of incorporation of different substituents on starch chains (Bronkowska, Orzel et al. 2013). Types of modification include esterification, etherification, and cross bonding (Nugent 2005). Resistance of RS4 starches increases with increasing substitution or chemical modifications. These modifications can hinder the interaction between enzyme and starch due to the compositional and structural changes made to the starch (Leszczynski 2004, Bronkowska, Orzel et al. 2013).

Effects that can alter or inhibit amylase activity can also disturb digestibility. Ways to alter amylase activity include the formation of amylose-lipid complexes and the incidence α-amylase inhibitors. Degree of chewing and intra-individual variations in transit time can also alter digestibility of starches (Nugent 2005).

Table 1. Descriptions and Sources of Resistant Starch

Type of Resistant Starch	Description	Sources
RS1	-Physically inaccessible to digestion. - Chewing or milling can mitigate resistance. - Heat Stable.	- Whole grains. - Seeds.
RS2	- Starch in its native granular form. - Food processing and cooking can reduce resistance. - Ungelatinized. - Typically has a high amylose level.	- Raw Potatoes. - Green bananas.
RS3	- Retrograted starch. - Non-granular starch.	- Cooked and cooled potatoes.
RS4	- Chemically or physically modified. - Examples of modifications: esterification, etherification, and cross	- Food products like breads, drinks, and others.

Table 1. Descriptions and Sources of Resistant Starch (continued)

	bonding. - Resistance increases with increasing chemical modifications.	

Resistant Starch: Methods of Measurement

The Association of Official Analytical Chemists (AOAC) Official Method 991.43 was used for determining insoluble dietary fiber in the starches, to determine the percent resistance. The benefit to the AOAC 991.43 method is that it can directly measure insoluble dietary fiber, which is correlated to the resistant starch available in the sample. Thought to mimic human digestion, this method provides a sequential enzymatic digestion by a heat stable α-amylase, followed by protease, and finally by amyloglucosidase.

α-amylase is the first subjection to enzymatic hydrolysis to the food or starch sample. α-amylase cleaves α-1,4 linkages at random locations. The sample is incubated with the amylase for 15 minutes at 100°C. Protease removes any protein in the sample through hydrolysis of the peptide bonds in the protein chain. The protease stage lasts 30 minutes at 60°C. The solution is next adjusted to a pH of 4.0-4.7. In starches, it is typically not necessary to change the pH of the solution at this step. Amyloglucosidase, the last part of the digestion, is used to cleave the leftover α-1,6 and end α-1,4 linkages of terminal linkages of amylose and amylopectin. The condition is held for 30 minutes at 60°C. The sample is further filtered and the

residue is washed with a series of water, ethanol, and acetone, and then vacuum dried to yield insoluble dietary fiber. (1992, Zhang, Dhital et al. 2013)

Resistant Starch: Glucose and Insulin Response

Carbohydrate digestion impacts glucose absorption in the body, and in effect, changes the glycemic and insulin response (Brites, Trigo et al. 2011). Since RS releases glucose slowly, a portion of glucose escapes the small intestine; it would be expected to correlate with less glucose absorption in the organ. This would significantly lower insulin response in the body, due to the lessened amount of free glucose molecules released from hydrolysis of starch (Asp, Van Amelsvoort et al. 1996). Along with lowering the glucose response, RS could also help to maintain regular glucose levels in the blood, proving to be beneficial to a variety of chronic diseases, including diabetes (Brites, Trigo et al. 2011). The metabolism of RS occurs five to seven hours after consumption in the ileum and colon, whereas normal starch is digested almost straightaway (Fuentes-Zaragoza, Sanchez-Zapata et al. 2011). A longer digestion time could not only alter glucose response, but also insulin response and even satiety.

Insulin is a hormone that enables glucose uptake in muscle and adipose cells. It stimulates the storage of glucose in the form of glycogen by increasing the activity of glycogen synthase, the rate-limiting enzyme of glycogen synthesis (Cohen, Nimmo et al. 1978). Glucose uptake by cells would cause a decrease of glucose in the blood. Insulin plays other roles in the inhibition of the use of stored fat and signaling of

hunger and satiety, with a lessened degree of insulin response associated with a higher satiety (Holt and Miller 1995, Nugent 2005).

RS foods are more difficult to digest and in effect release glucose more slowly. The outcome of the slowed release of glucose is a lower blood glucose level, which in turn lowers insulin response (Nugent 2005, Sajilata, Singhal et al. 2006). Other proposals of the affect of RS on glucose include: RS inhibits α-amylase or increases the viscosity of stomach and small intestine contents (Ou, Kwok et al. 2001).

Raben et al (1994) discovered that after a test meal of RS, no stimulation or a modest stimulation of glucose and insulin occurred. Five male subjects were subjected to two test meals, consisting of 50g raw potato starch (RS2) or pregelatinized potato starch. Subjects experienced a glucose response nine times greater with the pregelatinized starch meal than with the RS2 meal. Insulin response after the digestible starch meal had increased by a factor of six from fasting insulin. A modest increase in insulin response after the RS test meal was observed, correlating with the finding that the RS mitigated normal postprandial glucose and insulin responses (Raben, Tagliabue et al. 1994). This effect has been seen in numerous studies. Robertson et al (2003) demonstrated the acute ingestion of a high-RS diet changed insulin sensitivity and clearance in a positive manner (Robertson, Currie et al. 2003). Johnston et al (2010), Haub et al (2010), Behall (1989), and Nilsson et al (2008) established similar results. Nilsson et al (2007) proposed that the glucose and insulin response in a breakfast meal after a RS evening meal would be significantly lowered. Fasting blood glucose was not

significantly different, but glucose response after the breakfast meal was significantly lowered with subjects that had been fed a resistant starch diet the evening before (Behall, Scholfield et al. 1989, Nilsson, Ostman et al. 2007, Nilsson, Ostman et al. 2008, Haub, Hubach et al. 2010, Johnston, Thomas et al. 2010).

Similar results have been found in rats. Bronkowska et al (2013) subjected Wistar rats to four diets: one control diet containing soybean oil, a second control diet containing lard, and two RS supplemented diets with the respective fat sources. The study lasted 28 days. Plasma glucose was lower in RS4 fed rats than in their perspective control diets (Bronkowska, Orzel et al. 2013).

However, some studies have found no significant difference in insulin and glucose response. In a crossover study, subjects received four meals, only differing in RS content, each within a week of one another. The test meals contained from 0% to 10.7% RS2, a high amylose starch, as a percentage of total carbohydrate in the test meal. There was no difference in postprandial glucose or insulin response for any dose of starch examined. Higgins et al (2004), Jenkins et al (1998), and Nestel et al (2004) found no change in postprandial insulin and glucose response. This could be attributed to fat content of the diet, and source of resistant starch (Jenkins, Vuksan et al. 1990, Higgins, Higbee et al. 2004, Nestel, Cehun et al. 2004). Table 2 summarizes studies that have compared resistant starch to glucose and insulin response.

These studies indicate a lowered glucose and insulin response due to consumption of RS (Table 2). Nilsson et al (2007) did not observe a significantly lowered response with RS doses of 11.5g or lower. Further research should be

considered to determine a minimum amount of RS to consume long-term to produce a significant lowering of glucose and insulin response. Haub et al (2010) observed that a RS4 meal contributed to a lower glucose response than that of a RS2 meal, both of which were significantly lowered compared to the control. This suggests that in humans, different resistant starches elicit different glucose responses. Further research is necessary to determine the exact effects of the different RS subtypes on glucose response. Overall, these results suggest that long-term and short-term intake of RS can improve upon glucose and insulin response.

Table 2. Studies of the effects of RS on glucose and insulin.

Author(s)	Subjects	Model Design	Parameters Measured	Results
Behall, Scholfield et al. (1989)	Human subjects: 12 males	Cross over study. Subjects were subjected to either 70% amylose or 70% amylopectin in starch diet, in which starch contributed to 34% of caloric intake, for 5 weeks. Fasting blood was drawn each week, and a glucose tolerance test was administered after four weeks of diet consumption.	Fasting blood was analyzed for glucose, insulin, triglycerides, cholesterol (total and HDL), urea nitrogen, and uric acid. Postprandial plasma was analyzed for glucose, insulin, and glucagon.	- No significant differences were observed for glucose and insulin for the glucose tolerance test following a normal meal. - Glucose and insulin response were lowered for the glucose tolerance test after a high amylose meal after 5 weeks on each starch.
Haub, Hubach et al. (2010)	Human subjects: 4 male, 7 female	Single dose meal consisting of 30 g carbohydrate or starch.	Fasting blood glucose, and glucose 30, 60, 90, and 120 minutes post	- Peak glucose concentration occurred at 120 minutes for RS4

Table 2. Studies of the effects of RS on glucose and insulin (continued)

			meal.	meal, and at 30 minutes for dextrose and RS2 meal. - AUC for glucose response was significantly lowered in RS2 and RS4 meals compared to control. - AUC for glucose response was significantly lowered in RS4 meal compared to RS2 meal.
Nilsson, Ostman et al. (2007)	Human subjects: 11 male, 6 female	Subjects consumed evening meals of different RS content. After a fasting period after the evening meal, a standardized	Blood was collected for analysis of glucose, insulin and various other parameters.	- Fasting blood glucose was not significantly different between evening meal treatment. - Subjects who

Table 2. Studies of the effects of RS on glucose and insulin (continued)

		breakfast was given to subjects.		consumed evening meals with more than 11.5 g/serving dietary fiber and resistant starch exhibited a lowered glucose and insulin response following a standardized meal the following morning.
Johnston, Thomas et al. (2010)	Human subjects: 12 male, 8 female	Subjects consumed a RS (40g) or placebo supplements daily for 12 weeks.	Insulin sensitivity was measured.	- Insulin sensitivity improved with resistant starch supplementation compared to control.
Raben, Tagliabue et al. (1994)	Human subjects: 10 male	Subjects consumed meal with or without RS. Meals consisted of 50 g total starch, where	Postprandial plasma glucose and insulin.	- Postprandial glucose and insulin were significantly lower after RS meal.

Table 2. Studies of the effects of RS on glucose and insulin (continued)

		RS contributed to 54% to the test meal.		
Al-Tamimi, Seib et al. (2010)	Human subjects: 6 male, 7 female	Subjects consumed a dextrose meal bar, a puffed wheat bar (34g), or a RS4 bar (34g).	Blood glucose and insulin were measured at 10, 20, 30, 60, 90, and 120 minutes after the meal bar consumption. Results were reported as AUC.	- Peak glucose and insulin levels were significantly lower after RS4 treatment compared to control treatment.
Bodinham, Frost et al. (2010)	Human subjects: 20 males	Subjects consumed meals containing 48g RS or placebo.	Postprandial blood glucose and insulin were measured every 30 minutes for 7 hours.	- No significant differences were observed for blood glucose. - Postprandial insulin was significantly lower after the RS meal.
Bronkowska, Orzel et al. (2013)	Rats: 32 male Wistar	Diets contained 33% RS4 and had differing amounts of fat. Diets	Blood was collected via cardiac puncture for determination of	- The RS4 diets demonstrated a significantly lowered

Table 2. Studies of the effects of RS on glucose and insulin (continued)

		were fed for 28 days.	glucose.	glucose results compared to the control.
Brites, Trigo et al. (2011)	Rats: 36 male Wistar	Rats were fed breads consisting of 20% RS + wheat flour, wheat flour,	For the last three days of the study, rats were tested for postprandial	- Rats fed the 20% RS + wheat flour bread displayed a significantly
		20%RS + maize flour, or maize flour for 21 days.	glucose response. After a 12-hour fast, rats were fed 2g of diet and blood samples were taken from the tail vein before meal consumption, and at 40, 100, and 160 minutes after the meal.	lowered glucose response to a meal compared to the other treatments. - No significant difference was observed for glucose response in the 20% RS + maize flour diet.
Kim, Chung et al. (2003)	Rats: Sprague-Dawley	Rats were fed diets containing 53% cornstarch, 23%	Fasting plasma glucose and insulin were measured through	- No significant differences were observed in fasting

Table 2. Studies of the effects of RS on glucose and insulin (continued)

		cornstarch + 30% RS from corn, or 23% cornstarch + 30% RS from rice for three weeks.	cardiac puncture after euthanasia.	insulin. - There was a tendency to decrease blood glucose in RS from rice diets, however this was not significant.

Resistant Starch: Cholesterol and Triglycerides

The liver plays a principal role in the maintenance of cholesterol homeostasis in humans. Low-density lipoproteins are taken up by the liver, degraded to bile acids, and excreted from the body (Soral-Smietana and Wronkowska 2004). Elevated levels of LDL cholesterol is linked with cardiovascular disease risk. Elevated triglycerides may be involved in the pathogenesis of atherosclerosis, which also increases cardiovascular disease risk (Fernandez, Roy et al. 2000).

RS can help regulate cholesterol metabolism in the liver by converting higher levels of lipids to bile acids for excretion. RS has been thought to reduce LDLs and VLDLs in the body (Bronkowska, Orzel et al. 2013). Lower cholesterol levels due to RS could be caused by increased bile excretion, lower cholesterol absorption, and the synthesis of SCFAs, which in turn lower cholesterol synthesis in the liver (Vanhoof and De Schrijver 1998, Fernandez, Roy et al. 2000).

Several proposals have been suggested for the underlying mechanism of the cholesterol lowering effect, including: inhibition of 3-hydroxy 3-methylglutaryl CoA reductase, the rate limiting enzyme of cholesterol synthesis, or propionate enhancement of bile acid excretion by binding to starch granules and cholesterol 7α hydroxylase activity (Chezem, Furumoto et al. 1997, Arora, Sharma et al. 2011). The effects of RS on triglyceride levels are thought to be due to the increased production of fatty acids in the cecum. The absorption of the fatty acids reduces the activity of regulatory enzymes of fatty acid synthesis (Morand, Levat et al. 1994).

Bronkowska et al (2013) studied the effect of RS4 in high fat diets. Four diets were tested, soybean oil without resistant starch, soybean oil with RS, lard with

cholesterol added, and lard with cholesterol and RS added. Serum levels of total cholesterol were significantly lower in both diets supplemented with RS4. Levels of triglycerides were reduced similarly. Liver analysis showed that total cholesterol was reduced for both RS4 diets, and both RS4 diets showed higher HDL levels (Bronkowska, Orzel et al. 2013). Lopez et al (2001) determined that rats fed with RS had lower cholesterol in stool than control groups, as well as enhanced bile acid and cholesterol excretion. This could show that resistant starch may promote the conversion of cholesterol to bile acids for excretion (Lopez, Levrat-Verny et al. 2000). Fernandez et al found lowered triglyceride and LDL cholesterol levels in guinea pigs fed with a diet supplemented with RS(Fernandez, Roy et al. 2000). Behall et al (1989) performed a study to determine the effects of amylose content on triglyceride, cholesterol levels, and other markers. Diets higher in amylose resulted in significantly lower total cholesterol and triglycerides. RS, which is higher in amylose than amylopectin, should show a similar effect (Behall, Scholfield et al. 1989). Table 3 shows a summary of studies in which RS affected lipid metabolism.

Through fermentation in the large intestine, RS produces of short chain fatty acids. Propionate, a primary SCFA produced via fermentation of RS, is thought to attenuate cholesterol synthesis in the liver, while increasing HDL production (Soral-Smietana and Wronkowska 2004). SCFA absorption is proposed to reduce the activity of fatty acid synthesis, which can decrease the production of triglycerides (Morand, Levat et al. 1994). The aforementioned studies performed upon rats showed a significant decrease in plasma total cholesterol levels with RS supplemented diets. Chezem et al (1997), however, observed a difference in total

cholesterol levels between different types of resistant starches, RS3 and RS4. Higgins et al (2004) found no significant effect of RS2 on blood triglycerides at doses of 10.7% RS or below. Further research should be considered to examine the effects of the different subcategories of RS on cholesterol levels. An analysis to determine the minimum amount of RS that needs to be consumed for a significant response should also be measured. Differing gut microflora profiles provide another possibility to the differences observed in the comparison of the studies. These studies do establish that long term dietary intake of resistant starches can maintain low serum lipids, which can be beneficial to cardiovascular and overall health.

Table 3. Resistant starch studies on lipid metabolism.

Author(s)	Subjects	Model Design	Parameters Measured	Results
Behall, Scholfield et al. (1989)	Human subjects: 12 males	Cross over study. Subjects were subjected to either 70% amylose or 70% amylopectin in starch diet, in which starch contributed to 34% of caloric intake, for 5 weeks. Fasting blood was drawn each week, and a glucose tolerance test was administered after four weeks of diet consumption.	Fasting blood was analyzed for glucose, insulin, triglycerides, cholesterol (total and HDL), urea nitrogen, and uric acid. Postprandial plasma was analyzed for glucose, insulin, and glucagon.	- Mean fasting triglyceride and cholesterol levels were significantly lowered in during the period in which the men ate the 70% amylose diet.
Higgins, Higbee et al. (2004)	Human subjects: 7 male, 5 female	Subjects received four meals differing in RS2 content, 0%, 2.7%, 5.4%, or 10.7% of total	Blood samples were taken at 0, 30, 60, 90, 120, 180, 240, 300, and 360 minutes after the	- Resistant starch had no significant effect on triacylglycerol levels, at any dose.

Table 3. Resistant starch studies on lipid metabolism (continued)

Brites, Trigo et al. (2011)	Rats: 36 male Wistar	Rats were fed breads consisting of 20% RS + wheat flour, wheat flour, 20%RS + maize flour, or maize flour for 21 days.	After a twelve hour fast, animals were euthanized and blood was collected for cholesterol and triglyceride analysis.	- The RS + wheat fed group and the RS + maize fed group displayed significant reductions in blood total cholesterol.
Kim, Chung et al. (2003)	Rats: Sprague-Dawley	Rats were fed diets containing 53% cornstarch, 23% cornstarch + 30% RS from corn, or 23% cornstarch + 30% RS from rice for three weeks.	Fasting plasma lipids were measured through cardiac puncture after euthanasia. Liver lipids were also extracted and determined.	- Both types of RS significantly lowered plasma total lipid and cholesterol concentrations compared to the control. - Total liver cholesterol was lowered in RS from rice fed rats compared to the control.

Table 3. Resistant starch studies on lipid metabolism (continued)

De Deckere, Kloots et al. (1993)	Rats: Male Wistar	Diets contained either a low or high amount of RS, with a control group fed guar gum.	Effects of RS on total cholesterol and triglycerides were measured.	- Rats fed with RS had lowered total cholesterol in a dose dependent manner. - Rats fed with RS had lowered triglycerides in a dose dependent manner.
Lopez, Levrat-Verny et al. (2000)	Rats: 64 male Sprague-Dawley	Rats were fed diets consisting of 20% RS in the form of raw potato starch or high amylose starch.	Blood was collected via cardiac puncture and analyzed for lipids.	- Rats that consumed resistant starches had lowered cholesterol absorption by 23%. - RS diets were also effective in lowering plasma cholesterol.
Fernandez, Roy et al. (2000)	Guinea pigs: Male Hartley	Diets consisted of 14% cellulose, 10% RS, or 1% cholestyramine for 4 weeks.	Plasma total cholesterol, HDL cholesterol, and triglycerides were	- Guinea pigs fed the resistant starch diet had lower plasma cholesterol than

Table 3. Resistant starch studies on lipid metabolism (continued)

			measured after euthanasia.	control. - No effects were observed for plasma triglycerides. - RS fed diets had lower LDL cholesterol levels than the control.

Resistant Starch: Fermentation and Short Chain Fatty Acid Production

Starches that reach the large intestine undergo bacterial fermentation. Diverse populations of bacteria found in the proximal colon are the primary organisms responsible for this starch fermentation. This fermentation in the gut yields end products such as hydrogen gas, carbon dioxide, methane and short chain fatty acids (Englyst, Kingman et al. 1996, Hijova and Chmelarova 2007).

SCFAs consist of 1 to 6 carbons, and are the preferred respiratory fuel of colonocytes. The principal SCFAs are acetate, propionate, and butyrate. In humans, acetate is present in the highest concentration, followed by propionate, then butyrate. More recent studies have introduced roles of SCFAs such as advantageous to ion transport, modulators of intracellular pH, cell proliferation and differentiation regulators, and regulators of gene expression (Cook and Sellin 1998).

Although all SCFAs can be used as fuel, butyrate is the favored respiratory fuel by colonocytes. Butyrate is converted to ketone bodies, which can be used as energy throughout the body (Scheppach 1994, Nugent 2005, Hijova and Chmelarova 2007). This respiratory energy produced can be used for microbial growth and maintenance, or for production end products that can be absorbed through the intestinal wall and circulated throughout the body (Topping and Clifton 2001). Fermentation of starch, therefore, can signify salvage of energy of indigestible food (Bullock and Norton 1999).

100-200 mM of SCFAs are produced daily, and are mostly absorbed by the colon (Cook and Sellin 1998). SCFAs are more rapidly absorbed in the colon when

the luminal pH is low, or when the levels of SCFAs are amplified. In an acidic environment, some intestinal bacterial pathogens are inhibited, which concludes that a lower pH would be beneficial for gut disease. SCFAs stimulate the absorption of water and sodium in the large intestine, which can in turn mitigate diarrhea (Soral-Smietana and Wronkowska 2004). The concentration of SCFAs decreases as following the lower digestive tract, found in the highest concentrations in the cecum and lowest in the distal colon (Cook and Sellin 1998). The site of resistant starch fermentation has been shown to potentially push more distally. As the distal colon is where most tumors arise, a low pH could be a major benefit to the protection and prevention against colon cancer by controlling cell growth, inhibiting bacterial pathogens, and controlling absorption. (Fuentes-Zaragoza, Sanchez-Zapata et al. 2011).

The liver utilizes acetate by converting it to acetyl-CoA, a precursor to the lipogenesis of long chain fatty acids and a stimulator of gluconeogenesis. Propionate is also metabolized in the liver, where it increases gluconeogenesis. Propionate has furthermore been found to attenuate cholesterol synthesis in the liver, as well as increase high-density lipoprotein production (Soral-Smietana and Wronkowska 2004).

Butyrate is metabolized in preference to glucose and glutamine by colonocytes as an energy-producing pathway in the form of ATP (Cook and Sellin 1998, Henningsson, Bjorck et al. 2001, Soral-Smietana and Wronkowska 2004, Hijova and Chmelarova 2007). The metabolism of butyrate stimulates cell migration and proliferation, making it an important substrate in the colonic mucosa in its

prevention of colon disease (Soral-Smietana and Wronkowska 2004). Butyrate has been shown by Clarke et al (2012) to induce higher rates of apoptosis in rats exposed to genotoxic agents. Apoptosis is an important process in the protection of damaged cells prone to malignancy (Clarke, Young et al. 2012). This result illustrates that butyrate possibly plays a role in the protection against colon cancer and disease.

Kleessen et al (2014) found that RS2 caused higher acetate and propionate concentrations than an RS1 diet and RS-free diet. Butyrate concentrations were higher in both the RS1 and RS2 diets than in the RS-free diet. The butyrate was of equal concentration for both RS diets (Kleessen, Stoof et al. 1997). Ferguson et al (2000) treated rats with different preparations of RS2 starches, which resulted in an increase in all SCFAs. It was concluded that some RS2 starches had a more significant increase in butyrate concentrations, which would promote those starches as more beneficial to gut health when regarding cancer. Butyrate is beneficial for colon cancer, as it has been found to reverse neoplastic changes (Ferguson, Tasman-Jones et al. 2000, Nugent 2005). Bullock et al (1999) found that addition of RS3 to the diet increased SCFAs proportional to dose (Bullock and Norton 1999). Langkilde et all (2002) performed a 24 hour in vitro study of raw green banana flour, which resulted in a increase of acetate and butyrate (Langkilde, Champ et al. 2002). These studies potentially show the potential benefit of RS on SCFA production, which would lead to improved gut health. Table 4 shows a summary of findings for RS effects on short chain fatty acids, particularly acetate, butyrate, and propionate.

Through fermentation in the large intestine, RS can produce SCFAs, including acetate, propionate, and butyrate. SCFAs are known to benefit gut health through a variety of mechanism, such as modulation of intracellular pH, ion transport, and gene expresion regulation (Cook and Sellin 1998). The following rat studies resulted in differing conclusions. Bullock et al (1999) observed a significant increase in SCFA production with increasing RS3 concentration, while Kim et al (2003) did not observe any significant differences. The reasons for the differing responses can be attributed to differing microflora profiles, or even type of RS. Investigation of the effects of different types of gut microflora and differing RS types should be considered to determine the specific effects of the parameters on SCFA production. These studies suggest that in humans, short chain fatty acid production, particularly acetate and butyrate, is increased when RS is consumed. Phillips et all (1995) found that SCFA concentration, mainly acetate and butyrate, increased in a dose dependent manner. However, only two doses of RS were tested. Further studies investigating the production of SCFA with different doses of RS should be considered, to determine a which doses can elicit a significant response.

Table 4. Studies of RS and fecal short-chain fatty acids.

Author(s)	Subjects	Model Design	Parameters Measured	Results
Langkilde, Champ et al. (2002)	Human subjects: 10 ileostomy subjects	Subjects were given a diet with 30g RS2 (raw green banana flour) or a cooked banana flour.	Ileostomy bags were changed every 2 hours, and contents were frozen for future analysis of SCFA.	- Acetate and butyrate concentrations were significantly higher in the RS2 diets than the control and cooked banana diets.
Phillips, Muir et al. (1995)	Human subjects: 5 male, 6 female	Subjects consumed differing diets consisting of different amounts of RS (5.0g/day or 39.0 g/day) for 3 weeks.	Stool was collected the third week of the study.	- Fecal concentration of acetate and butyrate were increased in a dose dependent manner.
Kim, Chung et al. (2003)	Rats: Sprague-Dawley	Rats were fed diets containing 53% cornstarch, 23% cornstarch + 30% RS from corn, or 23% cornstarch + 30% RS	At euthanasia, cecum contents were collected and immediately frozen. Contents were then analyzed for SCFAs.	- No significant difference was observed in SCFA concentration for all groups.

Table 4. Studies of RS and fecal short-chain fatty acids (continued)

		from rice for three weeks.		
Bullock and Norton (1999)	Rats: 42 male Wistar	Rats were fed seven different diets, ranging from 0% RS3 to 200% RS3.	Stool was collected from days 6 to 8, and gut contents were collected at euthanasia.	- Total SCFA production increased with increasing RS3 concentration.
Kleessen, Stoof et al. (1997)	Rats: 30 male Wistar	Rats were fed RDS, 16.7% RS2 + waxy maize, or 66.75 RS2 + waxy maize for 5 months.	Fecal samples were collected eight days, 1 month, 3 months, and 5 months after the start of the experiment.	- RS2 showed higher amounts of SCFAs, particularly acetate and propionate.
Nofrarias, Martinez-Puig et al. (2007)	Pigs: 16 pigs	Pigs were fed for 14 weeks on a diet consisting of raw potato starch (RS2) or cornstarch.	At euthanasia, proximal colon contents were collected and analyzed for SCFAs.	- Total SCFAs were not significantly different between treatments. - Acetate was larger in proximal colon for CS pigs than RPS pigs. - Butyrate was larger in RPS pigs than CS pigs.

Resistant Starch: Food Intake, Weight Maintenance, and Satiety

The increasing presence of obesity in North America is convincing the country to develop strategies to reduce body weight and food intake to combat the issue. The effect of RS on lowering food intake may support the possibility of an increased satiety. This reduction of food intake directly impacts body weight, which could prove to be beneficial in weight loss and management (Freeland, Anderson et al. 2009). Some research has proposed that high fiber foods, like RS, may increase gut hormone alterations, such as glucagon-like peptide 1 (GLP-1). GLP-1 is known to have physiological functions including increasing insulin secretion and decreasing glucagon secretion (Willis, Eldridge et al. 2009). Glucose level has been proposed to correlate with satiety. A low glucose response would signify lower food intake, which would show a greater satiety level.

Measurement of satiety in animals is difficult, if not impossible, so determining satiety is left to studies on humans. Freeland et al (2009) studied the effects of fiber in a breakfast meal to healthy adult males. Males were fed a preload of low fiber cereal, high fiber cereal, low fiber cereal with glucose and high fiber cereal with glucose. They were then monitored for food intake for the remainder of the day. Energy intake was lowered in the glucose supplemented meals and the high fiber cereal preload. Satiety was greater in high fiber diets compared to the low fiber diets (Freeland, Anderson et al. 2009). Willis et al (2009) found that of the different dietary fibers, RS showed a significantly greater satisfaction and fullness up to 120 minutes. These studies contribute to the concept of RS playing a key role in food intake and satiety, which could eventually lead to weight loss and control (Willis,

Eldridge et al. 2009). Nilsson et al (2007) found that after an evening meal of RS, subjects were more satisfied with a breakfast meal the following morning than the control (Nilsson, Ostman et al. 2007).

Brites et al (2011) showed that RS supplemented wheat diets yielded significant reductions in food intake, but did not significantly alter body weight in rats (Brites, Trigo et al. 2011). Aziz et al (2009) determined that obese rats fed RS had a significantly reduced energy intake (Aziz, Kenney et al. 2009). Bodinham et al (2010) completed a short-term study of resistant starch ingestion on food intake. Subjects consumed 48g of RS in test meals, and were required to keep a food log for 24 hours. Subjects that received the RS test meals consumed less food for the 24-hour period than the controls (Bodinham, Frost et al. 2010).

RS primarily causes satiety due to its indigestibility. Satiation is assumed to influence food intake, but the proportion of satiation and food intake is difficult to determine. However, if weight loss is the endpoint, a lowered food intake is a key, and additional studies are needed to examine the effects of RS on weight loss. It would be necessary to determine if the weight loss thought to be associated with RS is due to a reduced food intake, which would be due to a higher satiety, or another mechanism involving satiety signaling and hormones.

Resistant Starch: Adverse Effects

The consumption of RS has been associated with belching, flatulence, laxation, gas emission, nausea, and stomach pain (Grabitske and Slavin 2009). Heijnen et al (1996) completed a single blind study in which 27 males and 30

females were provided RS2 or RS3 supplements, totaling 30g daily for 3 weeks along with their normal food consumption habits. The results concluded that a dose of more than 30g/day of RS caused flatulence, bloating, belching, stomachache, and mild laxative effects (Phillips, Muir et al. 1995, Heijnen, Van Amelsvoort et al. 1996). Heijnen et al (1998) also performed a study in which 24 healthy men ingested a daily RS2 or RS3 supplement (32g/day) for 4 weeks in addition to their normal diet. 91% of subjects supplemented with RS3 and 82% of subjects supplemented with RS2 reported flatulence. Bloating was reported in 41% of RS3 supplemented subjects and 28% of RS2 supplemented subjects (Heijnen, Van Amelsvoort et al. 1998). Phillips et al (1995) completed a study in which 11 volunteers (5 male, 6 female) were subjected to a cross over study of high-RS diet (39g/day) or a low-RS diet (5g/day) for 3 weeks, where stool was collected and gastrointestinal symptoms were recorded. A significant level of flatulence was reported in participants fed a high-RS diet, concluding that at high doses, RS causes flatulence in humans (Phillips, Muir et al. 1995).

Gastrointestinal discomfort due to RS is not observed at lower doses, but some symptoms were observed at higher doses. These results conclude that at higher doses, such as 30g/day or higher, can cause gastrointestinal discomfort like flatulence and bloating. However, at these doses, additional gastrointestinal adverse effects were minimally reported. Additional research should be considered to determine which doses of RS can be consumed that do not cause gastrointestinal discomfort while still eliciting the positive effect on the aforementioned parameters.

Resistant Starch: Mitigation of Diarrhea

Diarrhea is an excessive loss of fluid in the feces. The benefit of starch to diarrheal disease can be contributed to increased fluid absorption through greater SCFA production. SCFAs stimulate the uptake of water and cations in the proximal colon. Examples of cations that SCFAs promote include sodium, calcium, magnesium, and potassium. These cations are commonly associated with decreased fluid loss due to diarrhea. Lopez et al (2001) observed an increase in absorption of zinc, magnesium, and calcium in rats fed resistant starch (Lopez, Levrat-Verny et al. 2000). Trinidad et al (1996) showed that calcium is absorbed in the colon, and the absorption is enhanced by increased SCFAs. It was also found that propionate stimulates calcium absorption at a higher level than acetate (Trinidad, Wolever et al. 1996).

RS improves stool consistency in diarrhea by isolating water from the liquid stool. The water holding capacity of RS allows for the absorption of the excess water characterized in diarrhea, ultimately increase fecal bulk (Bosaeus 2004). Cummings et al (1993) found that a significant increase in stool weight in subjects fed RS2 and RS3 (Cummings, Beatty et al. 1996). Along with the mitigating effects of fecal bulk on diarrhea, an increase in fecal bulk is also associated with the decreased incidence of colon cancer (Fuentes-Zaragoza, Sanchez-Zapata et al. 2011).

Minerals are absorbed by exchange with a hydrogen ion in the large intestine. SCFAs are protonated, and when they diffuse into colonocytes, they dissociate and release a proton. The dissociation of hydrogen stimulates a Na-H exchange, resulting in mineral absorption (Cook and Sellin 1998). The transfer of the hydrogen ion into the lumen would decrease the pH in the colon (Trinidad,

Wolever et al. 1996). A pH lowered by 0.5 units has been associated with a reduced risk of colon cancer (Brites, Trigo et al. 2011).

Lower pH in the colon promotes the fecal excretion of bile acids and neural sterols, because the lower pH lowers the solubility of secondary bile acids (Jacobasch, Schmiedl et al. 1999). Le Leu et al (2002) found that pH was lowered with a RS supplemented diet (Leu, Hu et al. 2002). Brites et al (2011) also found that diets supplemented with RS produced lower fecal pH than diets without RS, regardless of the type of starch mixed with the RS (Brites, Trigo et al. 2011).

SCFAs in the large intestine also promote blood flow through the viscera, which allows for more nutrient absorption, again decreasing diarrhea (Trinidad, Wolever et al. 1996). Increased blood flow could also promote cell proliferation (Cook and Sellin 1998).

SCFA has also been thought to limit the viability of cholera in the gut. It has been hypothesized that the bacteria adhere to the resistant starch granules, therefore removing the bacteria from the infection site. Topping et al (2003) found that total coliforms and *E. coli* lowered in the proximal colon after exposure to amylose starch (Topping, Fukushima et al. 2003).

To summarize, RS is thought to reduce diarrhea through SCFA production and its high water holding capacity. The proposed method for the reduction in diarrhea is that of increased stool bulk though a healthier gut flora. Gut flora increase and become more diverse through the production of SCFAs, the primary fuel source for these bacteria. The results mentioned in table 5 conclude a variety of findings. Explanations for these differences can be contributed to differing gut

microflora profiles, different tolerances to the starches, or differing water holding capacity between the resistant starch subtypes. Analyses looking further into microflora composition would be a good subject to explore to determine the mechanisms of RS on influencing the microflora, or its direct effects on gut barrier function through SCFA production.

Table 5. Studies of RS effects on diarrhea.

Author(s)	Subjects	Model Design	Parameters Measured	Results
Nofrarias, Martinez-Puig et al. (2007)	Pigs: 16 pigs	Pigs were fed for 14 weeks on a diet consisting of raw potato starch (RS2) or cornstarch.	At euthanasia, proximal colon contents were collected, weight, and measured for starch content.	- Colon content was significantly heavier in resistant starch fed pigs than control. - Upon analysis of starch, more starch was found in the proximal colon of the RS2 fed pigs than control.
Bhandari, Nyachoti et al. (2009)	Pigs: 84 piglets	Piglets were subjected 7% RS or 14% RS to treat post-weaning diarrhea.	Stool consistency was measured daily.	- 7% RS treatment improved stool consistency, however, 14% RS did not.
Cummings, Beatty et al. (1996)	Human subjects: 7 male, 5 female	Subjects consumed diets consisting of 17-30g/day of RS for 15 days.	Stool was collected, weighed, and then freeze-dried to constant weight.	- Stool weight/bulk was significantly increased in RS diets.

Table 5. Studies of RS effects on diarrhea (continued)

Ramakrishna, Subramanian et al. (2008)	Human subjects: 50 males	Oral rehydration therapy was admitted at 50g/L RS.	Total diarrhea fecal weight was measured as well as duration of diarrhea.	- High amylose maize starch reduced diarrhea duration by 55%.
Raghupathy, Ramakrishna et al. (2006)	Human subjects: 183 children	Subjects were given oral rehydration with 50 g/L RS or glucose.	Stool consistency and weight were measured until the development of formed stool or until 72 hours past therapy.	- Formed stool was developed significantly faster in RS treated children.
Rabbani, Teka et al. (2001)	Human subjects: 62 boys	Subjects were given diets consisting of either 250 g/L green banana or 4 g/kg pectin, or a rice diet alone.	Stool weight, frequency, and consistency were measured.	- Subjects receiving pectin or banana recovered from diarrhea faster than control. - Subjects receiving RS treatment improved stool consistency significantly.

Resistant Starch: Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a disease that is chronic and without a medical cure. IBD is most commonly present in developed western countries, including the United States. There are approximately 2.2-14.3 new cases of colitis per 100,000 people per year and 3.1-14.6 new cases of Crohn's disease per 100,00 people per year. More developed countries, like the United States, have different lifestyles, diets, and environmental exposures that could be the underlying reason for the high incidence of IBD in these areas (Loftus 2004).

The trademark symptom of IBD is uncontrollable inflammation of the intestinal mucosa. Inflammation can occur in any part of the digestive tract. Unfortunately, in IBD, inflammation is not down regulated, so patients are chronically inflamed. Dysfunctional immune host response, pathogen infection, or a defective mucosal barrier potentially causes the immunoregulatory defects of IBD (Hanauer 2006).

Ulcerative colitis is a chronic disease in which there is ulceration of the colonic mucosa and submucosa. SCFA enemas are a type of treatment for ulcerative colitis in humans. It has been suggested that if resistant starch increased SCFA production, then it may be a useful treatment for inflammatory bowel diseases like colitis (Nugent 2005). Diversion colitis is an inflammatory disease characterized by changes in crypt abscesses, lymphoid hyperplasia, ulceration, edema, and other histological parameters (Cook and Sellin 1998). Symptoms of colitis include diarrhea, rectal bleeding, abdominal pain, constipation, loss of appetite, and weight loss.

IBD is associated with disruption of tight junctions in the epithelium of the gut, which can increase permeability of the lining. A normal lining protects against luminal microbes and antigens, and regulates activation of immune responses. In IBD, since the barrier is altered, bacterial products are able to cross the barrier and come in contact with immune cells. The immune cells will respond and cytokines will be produced, leading to the addition of inflammatory cells to the epithelium, creating inflammation (Hanauer 2006).

IBD alters some specific inflammatory and immune regulators. The activity of Nuclear Factor kappa B, a transcription factor in inflammatory responses and macrophage apoptosis, is increased in IBD. Activation of NF-κB yields the production of cytokines, growth factors, and metabolites of reactive oxygen, which facilitate inflammation and can contribute to tissue damage (Hanauer 2006).

RS has been reported to influence the production of pro-inflammatory cytokines and the expression of the receptors on T- and B-lymphocytes that trigger immune responses. This can be partially attributed to the favored SCFA, butyrate. Segain et al (2000) reported that butyrate can directly inhibit inflammatory responses through down regulation of NF-κB, which is commonly increased in cases of IBD (Segain, Raingeard de la Bletiere et al. 2000, Nugent 2005).

Increased SCFA production also decreases luminal pH. One outcome of a lower pH in the lumen signifies a lower the activity of 7α-hydroxylase, the enzyme associated with the rate limiting step of bile acid synthesis. Another outcome of a lower pH in the lumen is the inhibition of the transformation of primary to secondary bile acids, particularly cholate into deoxycholate. Deoxycholate inhibits

proliferation in the rat colitis model (Jacobasch, Schmiedl et al. 1999). Fernandez et al (2000) however, did not find a significant lower 7α-hydroxylase activity in guinea pigs fed RS2 (Fernandez, Roy et al. 2000).

Harig et al (1989) found that in patients with colitis subjected to SCFA edemas, endoscopic score improved significantly. After cessation of the treatment, the scores worsened. This study revealed a potential benefit of SCFAs to colon health, as the replacement of SCFAs improved inflammation (Harig, Soergel et al. 1989). Breuer et al (1991) conducted a study with patients with distal colitis. 90% of the subjects improved histologically after twice daily SCFA irrigations for 6 weeks (Breuer, Buto et al. 1991).

Oral rehydration therapy is a common treatment for diarrheal disease. Ramakrishna et al (2000) administered oral rehydration solutions containing 50 g resistant starch in adolescents and adults with cholera. Mean duration of diarrhea for patients was significantly lower with the resistant starch solution. This concludes that RS as a supplement in oral hydration therapy can help reduce fluid loss (Ramakrishna, Venkataraman et al. 2000).

In the study of Jacobasch et al (1999) rats induced with colitis were fed RS2 diets. Histological markers of inflammation and normalization were improved. Markers that improved included colonic cell proliferation, uptake of SCFA, and restoration of apoptosis (Jacobasch, Schmiedl et al. 1999, Nugent 2005). Moreau et al (2003) tested resistant starch on rats with dextran sodium sulfate induced colitis, and found improvements in histological observations (Moreau, Martin et al. 2003).

Typical treatments for inflammatory bowel disease and colitis include fecal bulking agents and fiber. This makes RS a prime choice for treatment of such a disease. The mechanism of RS on the improvement of gut barrier function and inflammation associated with IBD should be contemplated. This could provide a future dietary treatment for inflammatory bowel diseases, like colitis.

Citrobacter rodentium

Citrobacter rodentium (C. rodentium) is a murine attaching and effacing pathogen that is used in laboratory mice. It produces lesions indistinguisible from those of *Escherichia coli (E. coli)* (Higgins, Frankel et al. 1999). This pathogen attaches to enterocytes of host mice and efface the cell microvilli to produce diarrhea and inflammation (Guttman, Lin et al. 2009).

C. rodentium has a similar virulence to *E. coli*, however, fecal shedding was several orders of magnitude higher in *C. rodentium* than in *E. coli*. The duration of *C. rodentium* shedding was three to four weeks. Unlike *E. coli, C. rodentium* has reproducibly infected mice and has caused colonic disease (Borenshtein, McBee et al. 2008).

Clinical signs induced by *C. rodentium* include: dehydration, weight loss, coat ruffling, reluctance to move, diarrhea, and high mortality. These symptoms are similar to the characteristics of IBD. IBD patients are also at a high risk of colorectal cancer, and *C. rodentium* can cause a similar risk due to the hyperplasia of the mucosal lining (Borenshtein, McBee et al. 2008).

Citrobacter rodentium: Hyperplasia

C. rodentium causes epithelial hyperplasia in the distal colon. Hyperplasia is described as hyperproliferation of cells associated with NF-κB activstion(Borenshtein, Nambiar et al. 2007). As stated previously, activation of NF-κB produces growth factors, reactive oxygen metabolites, and cytokines, all of which contribute to inflammation and tissue damage. NF-κB can be activated in the following way: due to proinflammatory cytokines lead to the activation of IKK, which phosphorylates NF-κB-bound IκBs, releasing NF-κB to bind cytokines, chemokines, immunorecetors and other target genes (Borenshtein, Nambiar et al. 2007, Borenshtein, McBee et al. 2008). Increases in NF-κB activity due to *C. rodentium* have been seen in mice as early as 3 days postinoculation and increased through 12 days postinoculation. Wang et al (2006) found that on day 12 postinoculation, gland hyperplasia was at its maximum (Wang, Xiang et al. 2006).

Citrobacter rodentium: Development of Diarrheal Illness and IBD

Diarrheal illness leads to dehydration, which can be life threatening. Potential causes of diarrhea with *C. rodentium* include disruption of tight junctions resulting in impairment of intestinal barrier function, alterations in active transport, alterations in enteroendocrine serotonin signaling, and mucosal serotonin signaling. Gap junctions are key structures for the normal function of tissues. They provide intercellular channels for molecule movement. *C. rodentium* can cause changes in localization of aquaporins 2 and 3 (AQP2 and AQP3). AQP2 and AQP3 are water channels involved in water transport in intestinal epithelial cells. They conduct

water molecules through the cell and prevent the transport of unwanted ions. The changes due to *C. rodentium* can lead to water and electrolyte retention, in which the consequence is diarrhea (Borenshtein, McBee et al. 2008).

E. coli infection presents a significant health risk, especially in developing countries. Strains of *E.coli* can lead to diarrhea, dehydration, and death in some situations. A significant disease that can arise from *E. coli* is hemorrhagic colitis, which can be fatal. Causation of diarrheal illness in humans due to *E. coli* includes changes in the epithelium to lessen absorption, tight junction integrity loss, and permeability changes, all which lead to tissue damage. As stated above, *C. rodentium* has similar effects, allowing it to be a useful model for acute diarrheal disease and other gastrointestinal diseases like IBD. Mechanisms of *C. rodentium* infection are homologous to those with *E. coli* in humans, including the large number of bacterial attachment to the epithelial cell surface, thinning of the brush border, and epithelial extension beneath the bacteria (Luperchio, Newman et al. 2000, Lebeis, Bommarius et al. 2007).

IBDs, like colitis, can be mimicked by pathogens such as *C. rodentium*. The mechanisms behind this are thought to be disruption of tight junctions, alterations in active transport, and alterations in serotonin signaling in the mucosal lining of the gut.

Citrobacter rodentium: Host Defense

The *C. rodentium* infection is self-limiting, and it takes approximately 7 days for the bacteria to colonize. The infection takes about three to four weeks for

clearance. Most adult mice have been shown to have a lower morality rate with *C. rodentium* than younger mice (Luperchio, Newman et al. 2000). Fortunately, mice that recover from the *C. rodentium* infection have been shown to be resistant to the infection when further challenged (MacDonald, Frankel et al. 2003).

B- and T- cells are needed to survive infection with *C. rodentium* in mice. T-cell help results in B-cell maturation and IgG production. IgG has been shown to be protective against *C. rodentium* infection, as it can be transported across the epithelial barrier of the gut (Borenshtein, McBee et al. 2008).

Studies that involve infection via *C. rodentium* are shorter-term studies, due to the self-limiting property of the pathogen. Effects of the pathogen are also not likely to be shown until 7 days postinoculation. The drawback to this type of model is the limit of length that a study can be performed due to the time to onset and quick clearing of *C. rodentium.*

Citrobacter rodentium: Histological Changes

Generally accepted histological changes found in mice infected with *C. rodentium* include goblet cell loss, epithelial cell hyperplasia, and crypt elongation. These effects are commonly seen in inflammation (Luperchio, Newman et al. 2000). Borenshtein et al (2007) found that FVB mice developed substantial inflammation, edemas, and ulceration in the colon after inoculation of *C. rodentium*. The changes were most severe in the mid to distal colon and did not involve the cecum. Other changes seen in the *C. rodentium* infected mice included loss of goblet cell differentiation and dysplasia (Borenshtein, Nambiar et al. 2007). Hyperplasia is

associated with changes like crypt hyperplasia, crypt dilation, epithelial cell proliferation, mucosal height elevation, and an uneven apical enterocyte surface (Higgins, Frankel et al. 1999).

Higgins et al (1999) studied the histopathological results of the *C. rod* infection. By day 6, 60% of the mice experienced thickening of the distal colon. All mice experienced thickening by day 12. Epithelial cell hyperplasia was increased two to fourfold in the wild-type bacteria infected mice (Higgins, Frankel et al. 1999). To summarize, common histological changes to expect from the *C. rodentium* model are goblet cell loss, epithelial cell hyperplasia, crypt elongation, edema, and mucosal height elevation.

Study Overview

Purpose: The purpose of this study was to examine the effects of a chemically modified resistant starch, RS4, on diarrhea and inflammation induced by C. *rodentium*. We hypothesized that a diet supplemented with RS4, contributing to 25% resistance of the total starch in the diet (55% starch diet), would significantly improve stool consistency and provide protection against the inflammation associated with the *C. rodentium* pathogen, including inflammation score, mucosal height, ulceration, goblet cell loss, edema, and hyperplasia.

Design: 36 mice (18 male, and 18 female) were randomly assigned four treatment groups: uninfected mice fed the control starch diet, uninfected mice fed the RS4 supplemented diet, *C. rodentium* infected mice fed the control starch diet, and *C.*

rodentium infected mice fed the RS4 supplemented diet. After inoculation with *C. rodentium*, mice were be subjected to the diets for two weeks, and daily food intake, body weight, and stool consistency were measured. At the completion of the two weeks, mice were euthanized and blood was collected via cardiac puncture for serum glucose, insulin, and lipid analysis. Colon and cecum contents were collected and analyzed for pH, stool fat, and water content; and the tissues were sent for histopathology scoring.

Expected results: *C. rodentium* infected mice fed the RS4 supplemented diet were expected to show a significant increase in stool consistency compared to the infected mice the fed the control starch diet. The infected mice fed the RS4 diet were also expected to have a less severe inflammatory response due to the *C. rodentium* compared to the infected mice fed the control diet, which would be seen in the histopathology scores. Body weight loss and decreased food intake due to the *C. rodentium* pathogen was expected to be less severe in mice fed the RS4 supplemented diet, due to its expected protection against inflammation and diarrhea.

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CHAPTER III

TYPE 4 RESISTANT STARCH DIMINISHES *CITROBACTER RODENTIUM* INDUCED DIARRHEA IN C3H MICE

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ABSTRACT

Colitis is a chronic disease without medical cure, characterized by uncontrollable inflammation of the colonic mucosa and diarrhea. Resistant starch is a type of dietary fiber that is thought to improve upon stool consistency and inflammation via production of short chain fatty acids and absorption of water by the starch. *Citrobacter rodentium,* an A/E pathogen, mimics the inflammation and diarrhea associated with colitis. We hypothesized that a RS4 supplemented diet would reduce the severity of diarrhea and inflammation in the *C. rodentium* mouse model. C3H mice were inoculated with $5x10^8$ CFUs of *C. rodentium* (12 male, 12 female) or LB broth (6 male, 6 female). Two diets were tested, a RS4 supplemented diet contributing 25% resistance of total starch (12 *C. rodentium* infected, 6 uninfected) and a control cornstarch diet (12 *C. rodentium* infected, 6 uninfected). Stool consistency, body weight, and food intake were measured daily for 14 days. At euthanasia, colon and cecum contents were removed for analysis of pH, water content, and fat content; and the tissues were sent for histopathology scoring. *C. rodentium* infected mice fed the control diet exhibited significant weight loss compared to the *C. rodentium* infected mice fed the RS4 diet. Infected mice fed the

control diet displayed a significantly lowered food intake compared to the uninfected mice on the control diet. Infected mice fed the RS4 diet did not display a significant decrease in food intake compared to the uninfected mice fed the RS4 diet. A significant increase in stool consistency was observed for infected mice fed the RS4 diet compared to the infected mice fed the control diet. When the significance level was increased to 0.10, infected mice fed the RS4 diet showed significant improvement on ulceration/epithelial injury, gland hyperplasia, and goblet cell loss. The results indicate a RS4 supplemented diet can reduce the severity of diarrhea caused by the *C. rodentium* mouse model, as well as provide a partial protection on the associated inflammation. Additional research should be considered to investigate mechanisms of RS4 on inflammation and gut barrier function.

INTRODUCTION

Inflammatory bowel disease (IBD), including colitis, is a chronic disease without cure. Its trademark symptoms are diarrhea and uncontrollable inflammation of the intestinal mucosa. IBD is most commonly present in developed western countries, including the United States. The underlying reason behind the high incidence of IBD in these areas has been proposed to be due to differing lifestyles, diets, and environmental exposures (Loftus 2004).

The trademark symptoms of IBD, or colitis, involve diarrhea and uncontrollable inflammation of the intestinal mucosa. Inflammation can occur in any part of the digestive tract and is not down regulated, causing the inflammation to be chronic. Dysfunctional immune host response, pathogen infection, or a defective

mucosal barrier potentially causes the immunoregulatory defects of IBD and colitis (Hanauer 2006).

Resistant starch (RS) can be defined as any starch that resists digestion in the small intestine, and passes to the large intestine where it is subjected to fermentation (Englyst and Hudson 1996, Nugent 2005). RS is thought to be beneficial to gut health, through its fermentation in the large intestine to create short chain fatty acids. The production of short chain fatty acids is thought to improve gut barrier function, which can be beneficial to diarrheal and colonic diseases. In conjunction with the production of short chain fatty acids (SCFAs), RS has a high water holding capacity, thought to increase stool bulk and consistency, lessening the degree of water in stool. It has been suggested that if RS increases SCFA production, then it may be a useful treatment for inflammatory bowel diseases like colitis (Nugent 2005).

Citrobacter rodentium (C. rodentium) is a murine attaching and effacing pathogen that is used in laboratory mice. It produces lesions indistinguisible from those of *Escherichia coli* (Higgins, Frankel et al. 1999). This pathogen attaches to enterocytes of host mice and efface the cell microvilli to produce diarrhea and inflammation (Guttman, Lin et al. 2009). Clinical signs induced by *C. rodentium* include: dehydration, weight loss, coat ruffling, reluctance to move, diarrhea, and high mortality.

Potential causes of diarrhea with *C. rodentium* include disruption of tight junctions resulting in impairment of intestinal barrier function, alterations in active transport, and alterations in enteroendocrine serotonin signaling (Borenshtein,

McBee et al. 2008). Generally accepted histological changes found in mice infected with *C. rodentium* include goblet cell loss, epithelial cell hyperplasia, and crypt elongation. These effects are commonly seen in inflammation (Luperchio, Newman et al. 2000). Borenshtein et al (2007) found that FVB mice developed substantial inflammation, edemas, and ulceration in the colon after inoculation of *C. rodentium*. The changes were most severe in the mid to distal colon and did not involve the cecum (Borenshtein, Nambiar et al. 2007). The similarity of the gastrointestinal effects of *C. rodentium* and IBD allow for the *C. rodentium* mouse model to be suitable for examining potential treatments for mitigation of the chronic disease.

The purpose of this study was to examine the effects of a type-4 resistant starch (RS4) on diarrhea and inflammation in C3H mice triggered by *C. rodentium*, an A/E pathogen that causes inflammation and diarrhea similar to that of colitis. It was hypothesized that a diet supplemented with RS4 would primarily increase stool consistency in mice inoculated with *C. rodentium*. The RS4 diet treatment would also cause mice to experience a less severe inflammatory response due to the *C. rodentium*, which include goblet cell loss, mucosal height elevation, hyperplasia, edemas, and ulceration.

MATERIALS AND METHODS

Animals and Housing

Eight-week-old C3H mice, 18 male mice and 18 female mice, were purchased from Harlan Bioproducts (Indianapolis, IN). Animals were housed individually in micro-isolator cages. Each cage contained a raised wire floor, for determination of

stool consistency and stool weight. Mice were maintained on a 12-hour light/dark cycle in a temperature-controlled room. Temperature was maintained at 70°F. Water and food were provided *ad libitum* throughout the experiment. The animal studies were performed in compliance with the guidelines of the Iowa State University Institutional Animal Care and Use Committee (IACUC).

Diets

Two starches were evaluated in this study: a control starch, CS (Corn Starch CA 160170; Harlan Teklad, Madison, WI), and type-4 resistant starch, RS4 (RS-FiberGel60, DAESANG Corp., Seoul, Korea). Each starch was analyzed for resistant starch content, or insoluble fiber content, according to the AOAC method 991.43 for total, soluble, and insoluble dietary fiber. The RS4 was found to contain 69.3% resistant starch, and the control starch contributed 1.2% resistance.

Diets were a modification of the AIN-93G diet, in which total starch contributed 55% by weight of the total diet (Reeves 1997). The resistant starch diet was modified to reduce the resistance to approximately 25% insoluble fiber. This was accomplished by mixing the resistant starch with the control starch. Table 1 summarizes the resistant starch content for the starches and the mixture of the RS4 and control starch added to the diet. Diets were prepared according to Zhou et al., where starches were added by dry weight (Zhao, Hasjim et al. 2011). Diets were prepared daily, and were fed to the mice after a 24-hour drying period. Table 2 details the components of each diet, modified from the AIN-93G diet. Table 3

includes details for the caloric value of diet constituents, as well as grams of ingredient per kg diet.

Table 1. RS Content of Starches and RS Diet Mixture, According to AOAC 991.43

Starch Type	Resistant Starch Content (%)
Control Starch (CS)	1.2 ± 0.9
Resistant Starch (RS4)	69.3 ± 16
Diet Starch Mixture- RS4 + CS	25

Table 2. Diet Ingredients for the Control Starch Diet and the RS with Control Starch Diet.

Diet Ingredient	Control Starch Diet	RS4 Starch Diet
Control Starch (CS)	55%	~36.7%
Resistant Starch (RS4)	-	~18.3%
Casein	20%	20%
Dextrose	15%	15%
Mineral Mix (AIN-93)	3.50%	3.50%
Vitamin Mix (AIN-93)	1%	1%
Methionine	0.30%	0.30%
Choline	0.20%	0.20%
Corn Oil	5%	5%

Table 3. Caloric Value and g/kg in Control Diet and Resistant Starch Diet.

Diet Ingredient	CS Diet (g/kg diet)	CS Calorie Content (kcal/g)	RS4 Diet (g/kg diet)	RS4 Diet Calorie Content (kcal/g)
Control Starch (CS)	550 CS	2.2	~183 RS4	0.7 RS4
Resistant Starch (RS4)			~367 CS	1.5 CS
Casein	200	0.8	200	0.8
Dextrose	150	0.6	150	0.6
Mineral Mix (AIN-93)	3.5	-	35	-
Vitamin Mix (AIN-93)	10	-	10	-
Methionine	3	-	3	-
Choline	2	-	2	-
Corn Oil	50	0.45	50	0.45

Citrobacter rodentium culture

The initial culture suspension of *C. rodentium* (ATCC, DBS100, 51459; Manassas, VA) was diluted to a 10^5 dilution and incubated overnight at 37°C. Serial dilutions were made at factors of 10^6, 10^7, 10^8, and 10^9. LB agar plates were prepared in water, and 100uL of the dilutions of *C. rodentium* samples were streaked onto the plate. After overnight culture at 37°C, colonies were counted to calculate the CFUs in the stock solution. Based upon the counts, the stock solution contained $1.7x10^{13}$ CFU/mL stock solution. To make a solution of $5x10^8$ CFUs per 100 uL, the stock solution was diluted 10^4 with LB broth, and incubated overnight at 37°C. This final solution was used to inoculate the mice with 100 uL of solution by oral gavage.

Procedures

Mice were randomly assigned to the two test diets based upon weight, for a mean weight of 20.38 ± 1.72 grams and 28.6 ± 1.75 grams for females and males, respectively, in each diet group. Mice were allowed to acclimate for one week on a modified AIN-93G diet before being fed the test diets (Harlan Teklad, Madison, WI). Body weight was measured at the start and once at the end of the week acclimation period.

Mice were also assigned to *C. rodentium* treatment groups, infected or non-infected. Those assigned to the *C. rodentium* treated group were inoculated by gavage with 100uL of LB Broth containing 4-5x10^8 CFUs of *C. Rodentium* (Borenshtein, Nambiar et al. 2007). Mice that were placed in the non-infected group were gavaged with 100uL of LB Broth with no *C. Rodentium* in the suspension.

Body weight, food intake, water intake, and stool consistency were measured daily for 14 days after *C. rodentium* infection. Two days before euthanasia, stool was weighed along with measurement of stool consistency.

Mice were fasted for 12 hours prior to euthanasia. Fresh stool was collected immediately before euthanasia to be used for determination of pH and fat content. At euthanasia, blood was collected by cardiac puncture, approximately 0.5 mL per mouse. The blood was then allowed to sit for 30 minutes to 1 hour, centrifuged at 3500 rpm for 15 minutes in an Eppendorf 5418 Centrifuge (Eppendorf; Hamburg, Germany), and serum collected for analysis of glucose, insulin, and lipids. Serum was stored at -80° C before analysis. The colon and cecum of each mouse were removed,

and the contents collected and dried to determine water content. The tissues collected were stored in 10% formalin and analyzed for gut histopathology.

Blood Analysis

Fasting serum glucose was measured using YSI 2700 Select Biochemistry Analyzer (YSI Incorporated; Yellow Springs, OH). Fasting serum insulin was determined with Mercodia Ultrasensitive Insulin ELISA kit (Mercodia AB; Uppsala, Sweden). Serum lipids measured included: high-density-lipoprotein cholesterol, low-density-lipoprotein cholesterol, and triglycerides. Lipids were measured using Abnova HDL and LDL/VLDL standard kits and Abnova Triglyceride Quantification kit (Abnova; Taipei, Taiwan).

Stool Consistency, Fat, and pH

Stool consistency was graded on a 5-point scale, according to Hall et al (Hall, Melendez et al. 2013). A grade of 1 was assigned to feces that were not solid and were comprised of more than 75% liquid. A grade of 2 was given to feces that were soft and mounded, and that were consisting of 50% liquid. A grade of 3 was assigned to feces that had some cylindrical shape and more than 75% solid. A grade of 4 was assigned to feces that were more than 75% cylindrical and if more than 50% of the feces were firm. A grade of 5 was assigned to feces if the feces were cylindrical and more than 80% firm.

Stool samples were collected prior to euthanasia, and samples were measured out and mixed vigorously with water, 3 mL per 100 mg of colon content.

(Norman J. Temple and El-Khatib 1987) Stool pH was measured using a glass electrode and Corning pH/ion analyzer 350 (Corning).

Stool fat was measured using a modified method from a method determined by Bligh and Dyer (Bligh and Dyer 1959). 100 mg of frozen fecal sample was homogenized in a mixture of 100 mL chloroform and 200 mL methanol for two minutes, before an additional 100 mL of chloroform was added and blended for thirty seconds, and another addition of 100 mL of water. The homogenate was filtered, and filtrate was transferred to a graduated cylinder and allowed to separate completely. The chloroform layer was recorded, and the alcoholic layer was removed. The chloroform, or lipid, extract was evaporated by a stream of nitrogen and dried in a desiccator overnight. The total fat was calculated using the formula given by Bligh and Dyer (1959).

Histopathology

After removal, colons were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned longitudinally, and stained with hematoxylin and eosin. Sections were scored by Dr. Jesse Hostetter (Iowa State University, Department of Veterinary Pathology). Slides were viewed using an Olympus BX40 research/diagnostic grade microscope (Center Valley, PA).

The scoring system for histopathology used a scale from 0 to 5. A score of 0 was given when the parameter was absent. A value of 1 was assigned when the parameter was at a low level. A score of 3 was a parameter that was common and present in most high power fields. A 4 was given for a severe parameter that was

present in multiple events. The highest value, 5, was when the parameter was so severe and frequent that the structure of the tissue was distorted or lost.

Epithelial injury and ulceration was a measure of the damage to enterocytes in the lining of the gut or along the mucosal surface. The score was higher when ulceration was evident. Inflammation score indicated the density of inflammatory cells in the mucosa. A normal value for this score was 1, and in inflammation, the score increased. Edema signified tissue fluid expansion in the mucosa or submucosa. Stromal collapse indicated loss of glands in a region in which the mesenchymal stroma collapsed on itself. Gland hyperplasia specified excess proliferation in the tissue. Normal tissues had a score of around 0, and in inflammatory situations, the value increased. Goblet cell change was a marker of the decreases in goblet cell density in the lining of the glands. For this parameter, a value of 0 was a score for normal tissues, and increased with inflammation.

Statistical Analysis

Body weight and food intake were measured by mean values and ANOVA. Mean values were analyzed for fasting glucose, insulin, stool weight, stool consistency, stool weight per gram food intake, stool pH, and stool fat. Gut histopathology mean scores were analyzed. All values are reported as the mean ± standard deviation. All statistical analyses were performed using SPSS software (IBM), and $p<0.05$ and $p<0.10$ were considered to be significant.

RESULTS

Body Weight

No significant difference was observed for the change in body weight from baseline weight between all treatments for days 2, 3, 5, or 6 (Table 1A, Figure 1A). When separated by sex, no significant differences were observed between treatments for males on days 2, 3, 4, 5, or 6 (Table 1B, Figure 1B). For female mice, no significant difference was observed for the first 4 days, day 6, 7, or the last 4 days (Table 1C. Figure 1C).

C. rodentium infected groups fed the RS4 diet had a significant net loss in body weight compared to baseline on the first day (Table 1A, Figure 1A). When separated by sex, no significant difference between treatments was observed for day 1 for females (Table 1C. Figure 1C). However, a significant decrease in body weight from baseline was observed in male mice fed the RS4 diet compared to male mice fed the control diet (Table 1B, Figure 1B).

On day 4, the uninfected mice on the RS4 diet showed a significant difference in body weight compared to the *C. rodentium* infected mice fed RS4. Uninfected mice fed RS4 did not differ from the uninfected mice on the control diet (Table 1A, Figure 1A). This effect was not seen when body weights were analyzed by sex (Table 1B, 1C, Figure 1B, and 1C).

Uninfected female mice on the RS4 diet, on day 5, showed a significant difference in body weight change from baseline compared to the *C. rodentium* mice on the RS4 diet. The infected mice on the RS4 diet displayed an increase in body

weight from the baseline, whereas the uninfected mice had a decrease in body weight from baseline (Table 1A, Figure 1A).

Both infected on the RS4 diet and the infected mice on the control diet exhibited a net loss in body weight on day 7 compared to the uninfected mice on the control diet (Table 1A, Figure 1A). Male mice experienced the same significant weight loss for both infected mice groups compared to the uninfected control diet fed mice (Table 1B, Figure 1B). Females, however, did not display a significant weight difference between treatments (Table 1C, Figure 1C).

All mice showed a similar trend for changes in body weight on day 8 as for day 7, where there was a net loss in body weight for mice on both RS4 diets and the *C. rodentium* infected mice on the control diet. There was no difference in weight change between the two diets for uninfected mice (Table 1A, Figure 1A). When separated by sex, male mice that were infected tended to have a decrease in body weight compared to the uninfected mice on the control diet, although infected and uninfected mice on the RS4 diet were not significantly different from each other (Table 1B, Figure 1B). The female mice exhibited a decrease in body weight from baseline for infected and uninfected mice on the RS4 diet, and the infected mice on the control diet. The infected female mice on the RS4 diet had statistically similar body weights to both uninfected groups of female mice (Table 1C, Figure 1C).

The only mice that had a net increase in body weight on day 9 were the uninfected mice on the control diet. The infected mice on the control diet had a greater loss of body weight than the infected mice on the RS4 diet. The weight loss observed in both infected mouse groups, however, was not significantly different

than the body weight loss observed in uninfected mice on the RS4 diet (Table 1A, Figure 1A). In male mice, overall, a net loss of body weight was observed in the infected mice as compared to the uninfected mice. However, in infected vs. uninfected male mice fed the RS4 diet, the difference was not significant (Table 1B, Figure 1B). Female mice displayed a net loss in body weight for all groups except the uninfected mice on the control diet, but the net loss in body weight for both RS4 fed female groups were not significantly different than the uninfected control mice (Table 1C, Figure 1C).

On day 10, similar changes in body weights were observed for both groups of mice on the RS4 diet, and the uninfected mice on the control diet. A significant decrease in body weight was observed for infected mice on the control diet (Table 1A, Figure 1A). In male mice, the body weight changes on day 10 between *C. rodentium* infected mice on either diet were not statistically different, but the RS4 fed infected mice displayed a similar change in body weight compared to the uninfected mice fed RS4 (Table 1B, Figure 1B). These trends were not observed in female mice (Table 1C, Figure 1C). All mice on day 11 exhibited a similar change in body weight as day 10, but infected male mice on the control diet had a significant loss in body weight as compared to all other treatments (Table 1A, 1B, Figure 1A, 1B).

A significant loss of body weight was observed for infected mice on the control diet for both days 12 and 13 compared to the infected mice fed the RS4 diet. Uninfected mice fed the RS4 diet, showed a similar weight loss to the infected mice on the control diet (Table 1A, Figure 1A). Male mice did not display similarity

between the infected control mice and the uninfected RS4 mice. Instead, only the infected male mice on the control diet showed significantly lower body weight than other treatments (Table 1B, Figure 1B). These effects were not observed in the female mice.

Table 1A. Body weight change (g) from baseline by treatment.

Infection	Starch	N	Day 1 (g)	Day 2 (g)	Day 3 (g)	Day 4 (g)	Day 5 (g)	Day 6 (g)	Day 7 (g)	Day 8 (g)	Day 9 (g)	Day 10 (g)	Day 11 (g)	Day 12 (g)	Day 13 (g)
Uninfected	Control	12	0.1 ± 0.8^{ab}	-0.9 ± 1.6	0.2 ± 1.0	1.8 ± 2.6^{ab}	0.5 ± 1.1	0.7 ± 1.8	0.9 ± 1.6^{a}	1.2 ± 1.2^{a}	1.2 ± 1.4^{a}	0.8 ± 1.5^{a}	0.1 ± 1.4^{a}	0.3 ± 1.5^{a}	1.0 ± 1.5^{a}
Uninfected	RS4	11	-1.0 ± 0.3^{b}	-1.1 ± 0.7	-0.5 ± 0.6	-0.8 ± 1.0^{b}	-0.8 ± 1.1	-0.6 ± 1.5	-0.8 ± 1.3^{ab}	-0.6 ± 1.1^{ab}	-1.0 ± 1.3^{bc}	-0.7 ± 2.5^{a}	-0.7 ± 1.1^{a}	-1.1 ± 1.2^{ab}	-1.1 ± 1.6^{ab}
C. rod.	Control	6	0.2 ± 0.2^{a}	0.6 ± 1.1	0.8 ± 1.3	0.2 ± 1.1^{a}	0.0 ± 1.1	-0.2 ± 1.2	-1.1 ± 1.0^{b}	-2.0 ± 1.2^{b}	-2.7 ± 1.3^{b}	-2.9 ± 1.4^{b}	-2.9 ± 1.6^{b}	-3.0 ± 1.9^{b}	-2.5 ± 2.3^{b}
C. rod.	RS4	6	-0.9 ± 0.2^{b}	-0.8 ± 1.1	0.4 ± 0.8	0.3 ± 0.8^{a}	0.3 ± 1.0	0.7 ± 1.1	-0.3 ± 0.8^{b}	-0.8 ± 0.8^{b}	-1.0 ± 0.8^{c}	-0.6 ± 0.8^{a}	-0.6 ± 0.9^{a}	-0.7 ± 1.0^{a}	-0.3 ± 0.8^{a}
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Table 1B. Male body weight change (g) from baseline by treatment.

Infection	Starch	N	Day 1 (g)	Day 2 (g)	Day 3 (g)	Day 4 (g)	Day 5 (g)	Day 6 (g)	Day 7 (g)	Day 8 (g)	Day 9 (g)	Day 10 (g)	Day 11 (g)	Day 12 (g)	Day 13 (g)
Uninfected	Control	6	0.6 ± 0.1^{a}	0.4 ± 1.1	0.9 ± 0.6	1.4 ± 0.1	1.2 ± 0.5	1.6 ± 0.2	1.9 ± 0.2^{a}	1.8 ± 0.9^{a}	1.7 ± 0.7^{a}	1.6 ± 0.7^{a}	1.0 ± 0.7^{a}	1.1 ± 1.2^{a}	1.6 ± 1.4^{a}
Uninfected	RS4	5	-1.1 ± 0.3^{b}	-1.0 ± 0.2	-0.6 ± 0.8	-0.2 ± 0.7	0.1 ± 0.8	0.3 ± 1.7	0.0 ± 1.4^{ab}	0.3 ± 0.8^{ac}	0.0 ± 0.9^{ac}	0.7 ± 3.1^{a}	0.1 ± 0.3^{a}	-0.1 ± 0.6^{a}	0.2 ± 1.0^{a}
C. rod.	Control	3	0.6 ± 0.7^{a}	1.1 ± 1.2	0.7 ± 0.9	0.7 ± 1.1	0.5 ± 0.8	0.1 ± 1.0	-1.0 ± 0.7^{b}	-2.2 ± 0.9^{b}	-3.1 ± 1.2^{b}	-3.7 ± 1.1^{b}	-3.8 ± 1.5^{b}	-3.9 ± 1.4^{b}	-3.9 ± 2.0^{b}
C. rod.	RS4	3	-1.2 ± 0.8^{b}	-0.4 ± 0.9	0.2 ± 0.9	-0.2 ± 1.0	0.0 ± 1.2	0.2 ± 0.9	-0.7 ± 0.9^{b}	-1.2 ± 0.9^{bc}	-1.3 ± 0.9^{bc}	-0.9 ± 0.9^{ab}	-0.9 ± 0.9^{a}	-1.3 ± 1.1^{a}	-0.6 ± 1.1^{a}
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Table 1C. Female body weight change (g) from baseline by treatment.

Infection	Starch	N	Day 1 (g)	Day 2 (g)	Day 3 (g)	Day 4 (g)	Day 5 (g)	Day 6 (g)	Day 7 (g)	Day 8 (g)	Day 9 (g)	Day 10 (g)	Day 11 (g)	Day 12 (g)	Day 13 (g)
Uninfected	Control	6	-0.5 ± 0.8	-2.1 ± 0.6	-0.6 ± 0.3	2.3 ± 4.1	-0.1 ± 1.3^{ab}	-0.2 ± 2.4	-1.6 ± 0.4	0.6 ± 1.4^{a}	0.6 ± 1.9^{a}	0 ± 1.7	-0.9 ± 1.4	-0.6 ± 1.3	0.4 ± 1.5
Uninfected	RS4	6	-0.9 ± 0.3	-1.2 ± 1.1	-0.4 ± 0.6	-1.5 ± 0.8	-1.7 ± 0.2^{b}	-1.4 ± 0.4	-0.2 ± 1.9	-1.5 ± 0.5^{ab}	-2.1 ± 0.1^{ab}	-2.0 ± 0.6	-1.5 ± 1.0	-2.1 ± 0.8	-2.3 ± 0.7
C. rod.	Control	3	-0.3 ± 0.9	0.0 ± 0.9	0.8 ± 1.6	-0.3 ± 0.9	-0.5 ± 1.1^{ab}	-0.5 ± 1.5	-1.2 ± 1.3	-1.9 ± 1.4^{b}	-2.2 ± 1.3^{b}	-2.1 ± 1.2	-2.1 ± 1.3	-2.0 ± 1.9	-1.4 ± 2.0
C. rod.	RS4	3	-0.7 ± 0.8	-1.1 ± 1.2	0.5 ± 0.7	0.6 ± 0.5	0.5 ± 0.9^{a}	1.2 ± 1.1	0.0 ± 0.6	-0.5 ± 0.6^{ab}	-0.8 ± 0.7^{ab}	-0.3 ± 0.6	-0.3 ± 0.8	-0.3 ± 0.9	0.0 ± 0.7
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Figure 1A. Body weight changes (g) over time by treatment.

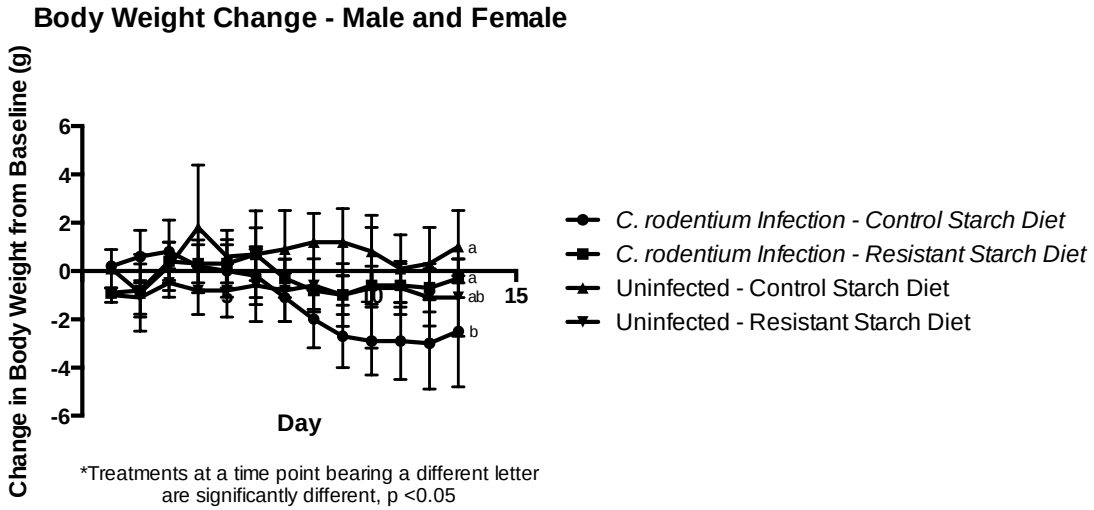


Figure 1B. Male body weight changes (g) over time by treatment.

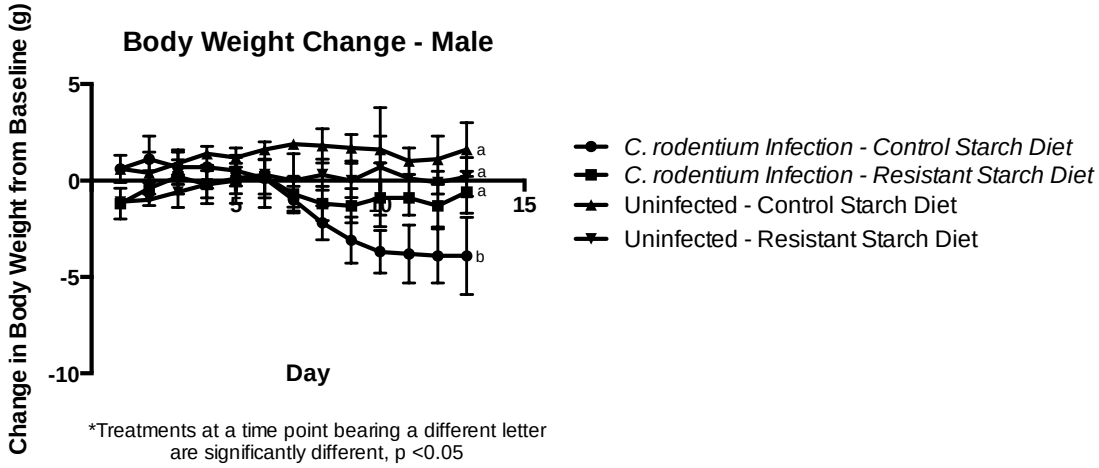
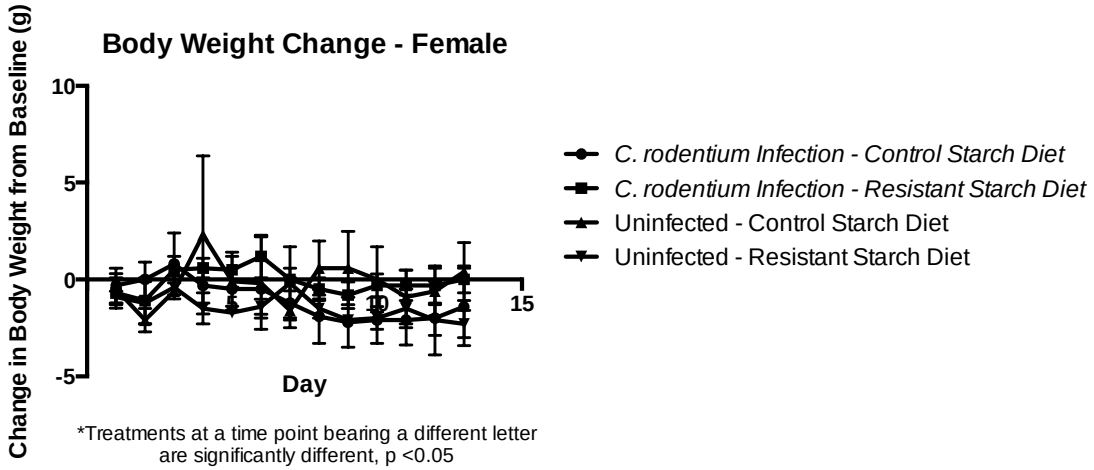


Figure 1C. Female body weight changes (g) over time by treatment.



Food Intake and Food Efficiency

No significant differences were observed in food intake for days 2, 3, 4, 5, 7, 8, 9, and 10. On day 1, RS4 fed mice that were not infected with *C. rodentium* showed a significantly lower food intake than the infected mice. This difference was not observed compared to the uninfected control starch fed mice. Infected mice on the control diet had significantly less food intake on day 6 than did the uninfected mice. Infected mice on the RS4 diet exhibited less food intake compared to the uninfected mice on the RS4 diet, but this was not observed compared to both groups fed control diet. On days 11 and 12, *C. rodentium* infected mice on the control diet had significantly less food intake compared to the infected mice on the RS4 diet, but no significant difference was observed between food intake for those mice and the uninfected groups. The RS4 fed mice that were infected with *C. rodentium* had an increased intake compared to the infected mice on the control diet, but no significant difference compared to uninfected mice. On the last day, there was a significant difference between food intakes for both RS4 diets, where the infected mice displayed a higher intake than those that were not infected (Table 2A, Figure 2).

In male mice, infected mice on the RS4 diet had a significantly decreased food intake compared to the uninfected mice fed the control diet on days 1 and 3. On day 6, a significant difference was observed in food intake between the uninfected RS4 fed mice and the infected control diet fed mice. On day 11, uninfected RS4 fed mice displayed a significantly lowered food intake compared to the RS4 fed infected mice. (Table 2B). In females, infected control fed mice showed a significantly lowered food intake on day 6 compared to uninfected control fed mice (Table 2C).

Food efficiency ratio is a measure of food intake and body weight output, or the mass gained in grams per gram of food intake. No significant difference was observed for food efficiency ratio for days 1-6 and 10-13. On day 7, no significant differences were observed when analysis was done without separating sexes (Table 3A, Figure 3A). Upon separation of sexes, males and females showed significantly different food efficiency in the uninfected mice on the control diet compared to all treatments (Table 3B, 3C, Figure 3B, 3C). On day 8, uninfected mice on the control diet showed a significantly higher food efficiency ratio than the infected mice on both diets (Table 3A, Figure 3A). In females, the uninfected RS4 fed mice had significantly higher food efficiency ratios than the infected mice, but this was not significantly different from the uninfected mice fed the control diet (Table 3C, Figure 3C). The same result for both sexes together on day 8 was also observed on day 9 (Table 3A, Figure 3A).

Table 2A. Food intake (g/day) by treatment.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Uninfected	Control	6	4.9 ± 1.8^{ab}	6.5 ± 1.3	7.6 ± 1.3	8.6 ± 0.6	7.2 ± 3	6.4 ± 1.3^{ab}	5.5 ± 2	7 ± 1.4	5.2 ± 1.4	8.9 ± 2	4.4 ± 2.3^{ab}	7.1 ± 1^{ab}	8 ± 2.4^{ab}
Uninfected	RS4	6	3.7 ± 0.9^{b}	5.8 ± 1.5	5.8 ± 0.9	8.7 ± 1	5.8 ± 0.8	6.7 ± 0.7^{b}	4.7 ± 1.1	6 ± 0.5	3.7 ± 0.8	9 ± 1.1	5.5 ± 1.5^{ab}	5.5 ± 1.7^{ab}	4.8 ± 2^{b}
C. rod.	Control	12	6.7 ± 1.6^{a}	5.6 ± 1.4	5.7 ± 1.6	9 ± 1.5	6.4 ± 2	4.6 ± 1.1^{c}	4.4 ± 1.8	5.8 ± 2.5	4.4 ± 2.5	8 ± 3.2	3.4 ± 1.6^{a}	5.5 ± 2.4^{a}	6.7 ± 2.2^{ab}
C. rod.	RS4	11	5.6 ± 1.5^{a}	5.5 ± 1.7	5.3 ± 1.5	8.5 ± 4	7.6 ± 2.4	5.5 ± 0.9^{ac}	5.1 ± 1	5.2 ± 1.6	3.3 ± 1.6	6.5 ± 2	8.3 ± 4.1^{b}	7.9 ± 1.6^{b}	8 ± 2.5^{a}
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Table 2B. Food intake (g/day) for male mice by treatment.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Uninfected	Control	3	5.9 ± 1.6^{a}	6.7 ± 1.6	7.9 ± 1.4^{a}	8.9 ± 0.8	8.4 ± 4.0	5.4 ± 0.4^{ab}	6.5 ± 1.7	6.5 ± 0.7	5.3 ± 1.9	9.2 ± 2.8	5.2 ± 1.3^{ab}	5.5 ± 1.9	7.0 ± 1.0
Uninfected	RS4	3	3.8 ± 1.4^{ab}	5.2 ± 1.5	5.9 ± 0.7^{ab}	8.8 ± 0.2	6.3 ± 0.6	6.8 ± 0.8^{a}	4.5 ± 0.7	6.1 ± 0.5	3.7 ± 0.3	8.6 ± 1.2	0.8 ± 7.7^{a}	6.4 ± 2.1	4.5 ± 3.2
C. Rod.	Control	6	7.5 ± 1.6^{ab}	5.4 ± 1.5	5.6 ± 1.4^{ab}	9.5 ± 1.0	7.0 ± 0.7	4.0 ± 1.1^{b}	4.4 ± 2.1	4.7 ± 2.3	4.1 ± 1.6	6.9 ± 2.4	3.0 ± 2.6^{ab}	4.4 ± 1.7	6.0 ± 2.2
C. Rod.	RS4	5	4.9 ± 1.6^{b}	5.4 ± 2.2	4.7 ± 1.4^{b}	11.2 ± 5.5	7.0 ± 1.6	5.1 ± 0.6^{ab}	5.0 ± 0.9	4.5 ± 1.3	3.4 ± 1.1	7.0 ± 1.8	10.4 ± 1.9^{b}	7.2 ± 1.2	6.8 ± 3.0
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Table 2C. Female food intake (g/day) by treatment.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Uninfected	Control	3	3.8 ± 1.4	6.3 ± 1.3	7.3 ± 1.3	8.4 ± 0.2	6.0 ± 1.2	7.4 ± 1.1[a]	4.6 ± 2.0	7.6 ± 1.9	5.1 ± 1.0	8.7 ± 1.6	3.5 ± 3.0	8.7 ± 1.7	9.2 ± 3.2
Uninfected	RS4	3	3.6 ± 0.3	6.5 ± 1.5	5.8 ± 1.2	8.6 ± 1.5	5.3 ± 0.7	6.6 ± 0.8[ab]	4.8 ± 1.6	5.8 ± 0.6	3.7 ± 1.1	9.3 ± 0.9	4.9 ± 0.9	4.5 ± 0.3	4.9 ± 0.3
C. Rod.	Control	6	5.9 ± 1.4	5.7 ± 1.4	5.8 ± 2.0	8.5 ± 1.8	5.8 ± 2.8	5.2 ± 0.7[b]	4.4 ± 1.7	6.9 ± 2.4	4.7 ± 3.3	9.1 ± 3.6	3.5 ± 1.4	6.7 ± 2.7	7.4 ± 2.2
C. Rod.	RS4	6	6.2 ± 1.3	5.6 ± 1.2	5.9 ± 1.6	8.2 ± 1.4	8.1 ± 2.9	5.8 ± 1.0[ab]	5.2 ± 1.2	5.7 ± 1.8	3.1 ± 2.2	6.0 ± 2.2	6.5 ± 3.0	8.5 ± 1.8	9.0 ± 1.7
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Table 3A. Food efficiency ratio (g food intake/g weight gained/day) by treatment.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Uninfected	Control	6	0.06 ± 0.05	0.02 ± 0.03	0.15 ± 0.14	0.20 ± 0.34	0.05 ± 0.06	0.05 ± 0.05	0.08 ± 0.13	0.07 ± 0.06[a]	0.02 ± 0.03[a]	0.00 ± 0.01	0.00 ± 0.00	0.04 ± 0.04	0.09 ± 0.03
Uninfected	RS4	6	0.00 ± 0.00	0.04 ± 0.05	0.14 ± 0.22	0.02 ± 0.03	0.05 ± 0.07	0.06 ± 0.08	0.01 ± 0.03	0.04 ± 0.05[ab]	0.01 ± 0.02[ab]	0.11 ± 0.15	0.19 ± 0.24	0.01 ± 0.02	0.03 ± 0.05
C. rod.	Control	12	0.05 ± 0.07	0.11 ± 0.15	0.08 ± 0.11	0.02 ± 0.04	0.02 ± 0.03	0.03 ± 0.05	0.02 ± 0.06	0.00 ± 0.00[b]	0.01 ± 0.02[b]	0.01 ± 0.03	0.12 ± 0.17	0.08 ± 0.19	0.08 ± 0.11
C. rod.	RS4	11	0.01 ± 0.02	0.12 ± 0.14	0.25 ± 0.28	0.02 ± 0.05	0.03 ± 0.03	0.11 ± 0.13	0.00 ± 0.00	0.01 ± 0.02[b]	0.01 ± 0.03[b]	0.12 ± 0.03	0.03 ± 0.05	0.03 ± 0.05	0.11 ± 0.15
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

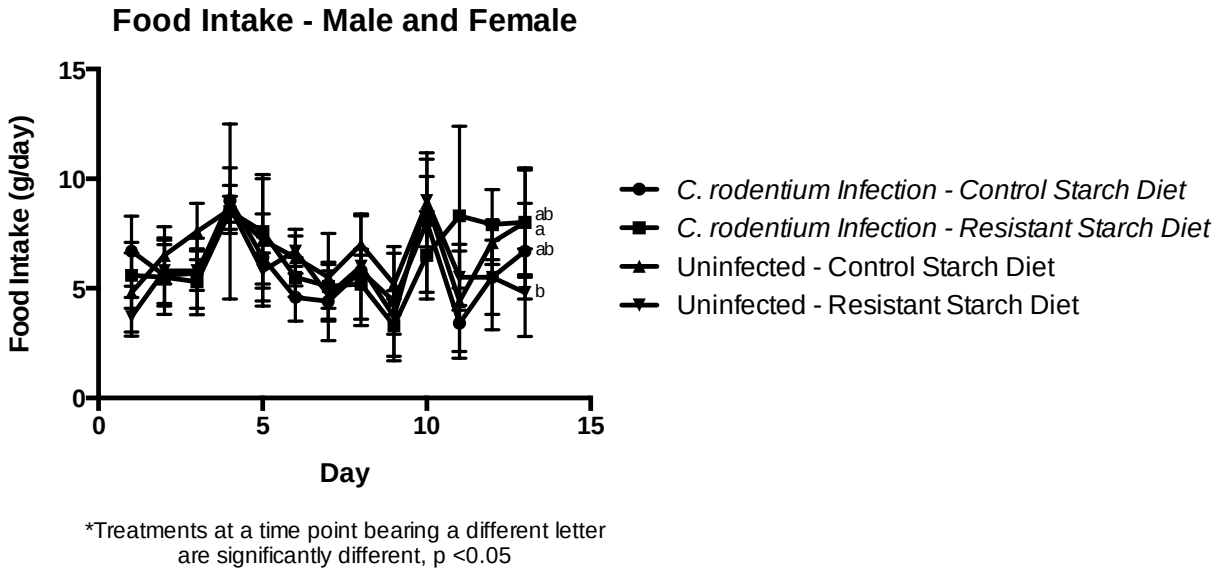
Table 3B. Male food efficiency ratio (g food intake/g weight gained/day) by treatment.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Uninfected	Control	3	0.10 ± 0.02	0.04 ± 0.03	0.08 ± 0.10	0.06 ± 0.06	0.02 ± 0.03	0.07 ± 0.06	0.05 ± 0.01[a]	0.04 ± 0.03	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.03	0.07 ± 0.02
Uninfected	RS4	3	0.00 ± 0.00	0.04 ± 0.07	0.08 ± 0.08	0.04 ± 0.03	0.05 ± 0.07	0.06 ± 0.06	0.05 ± 0.01[b]	0.04 ± 0.03	0.01 ± 0.02	0.19 ± 0.19	0.19 ± 0.23	0.01 ± 0.03	0.05 ± 0.06
C. rod.	Control	6	0.06 ± 0.07	0.15 ± 0.21	0.04 ± 0.08	0.04 ± 0.04	0.03 ± 0.05	0.00 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.22	0.12 ± 0.28	0.05 ± 0.05
C. rod.	RS4	5	0.00 ± 0.15	0.18 ± 0.15	0.18 ± 0.25	0.00 ± 0.00	0.03 ± 0.04	0.08 ± 0.10	0.00 ± 0.00[b]	0.00 ± 0.01	0.02 ± 0.04	0.08 ± 0.11	0.01 ± 0.02	0.05 ± 0.07	0.20 ± 0.20
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Table 3C. Female food efficiency ratio (g food intake/g weight gained/day) by treatment.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Uninfected	Control	3	0.02 ± 0.04	0.00 ± 0.00	0.22 ± 0.15	0.34 ± 0.48	0.07 ± 0.07	0.04 ± 0.05	0.11 ± 0.19[a]	0.11 ± 0.06[ab]	0.03 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.05	0.11 ± 0.04
Uninfected	RS4	3	0.00 ± 0.00	0.05 ± 0.04	0.19 ± 0.33	0.00 ± 0.04	0.05 ± 0.09	0.05 ± 0.05	0.02 ± 0.01[b]	0.02 ± 0.03[a]	0.00 ± 0.00	0.03 ± 0.10	0.20 ± 0.28	0.00 ± 0.00	0.00 ± 0.00
C. rod.	Control	6	0.03 ± 0.07	0.07 ± 0.06	0.12 ± 0.12	0.00 ± 0.01	0.01 ± 0.02	0.05 ± 0.06	0.04 ± 0.08[b]	0.00 ± 0.00[b]	0.02 ± 0.03	0.02 ± 0.03	0.04 ± 0.05	0.05 ± 0.06	0.12 ± 0.15
C. rod.	RS4	6	0.01 ± 0.02	0.07 ± 0.12	0.31 ± 0.30	0.04 ± 0.06	0.02 ± 0.03	0.14 ± 0.16	0.00 ± 0.00[b]	0.01 ± 0.02[b]	0.00 ± 0.00	0.15 ± 0.17	0.04 ± 0.06	0.02 ± 0.01	0.04 ± 0.03
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*															

Figure 2. Food intake (g/day) over time by treatment.



*Treatments at a time point bearing a different letter are significantly different, p <0.05

Figure 3A. Food efficiency ratio over time by treatment.

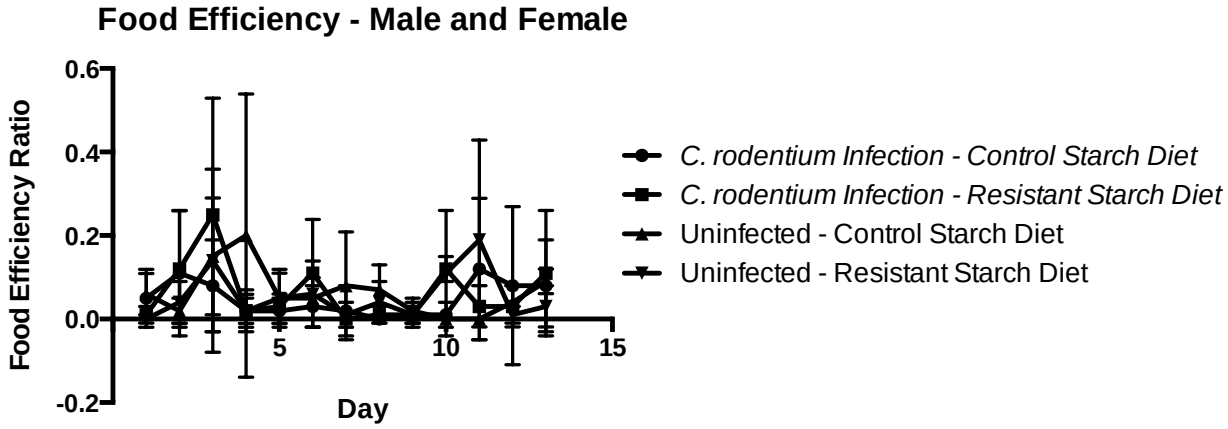


Figure 3B. Male food efficiency ratio over time by treatment.

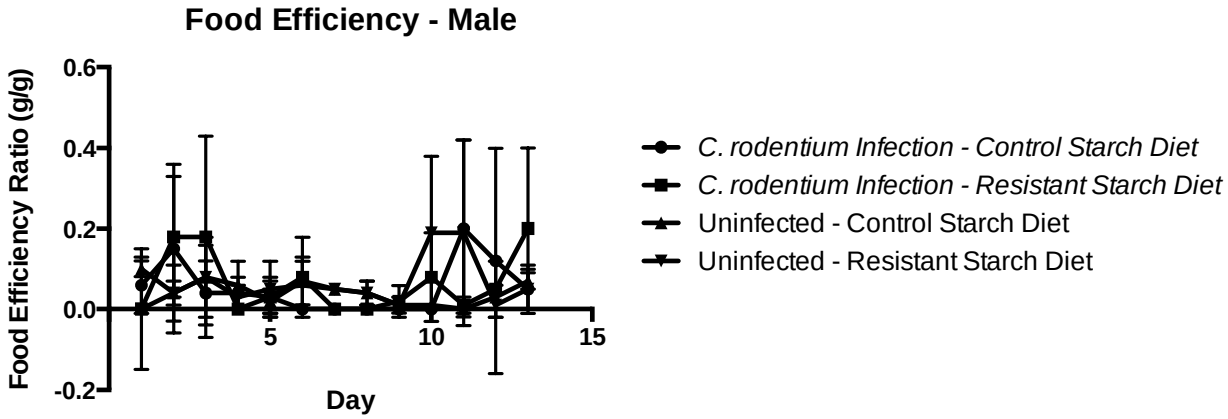
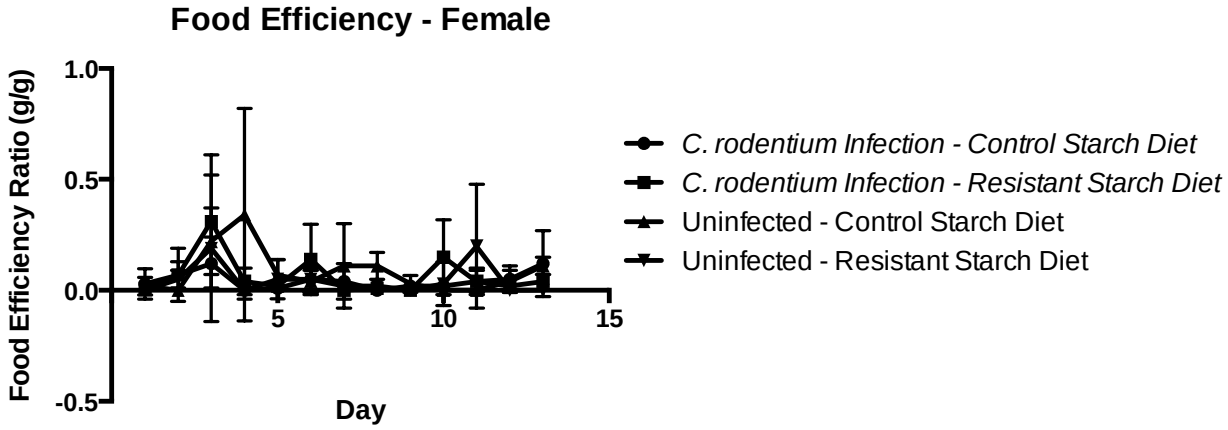


Figure 3C. Female food efficiency ratio over time by treatment.



Stool Consistency

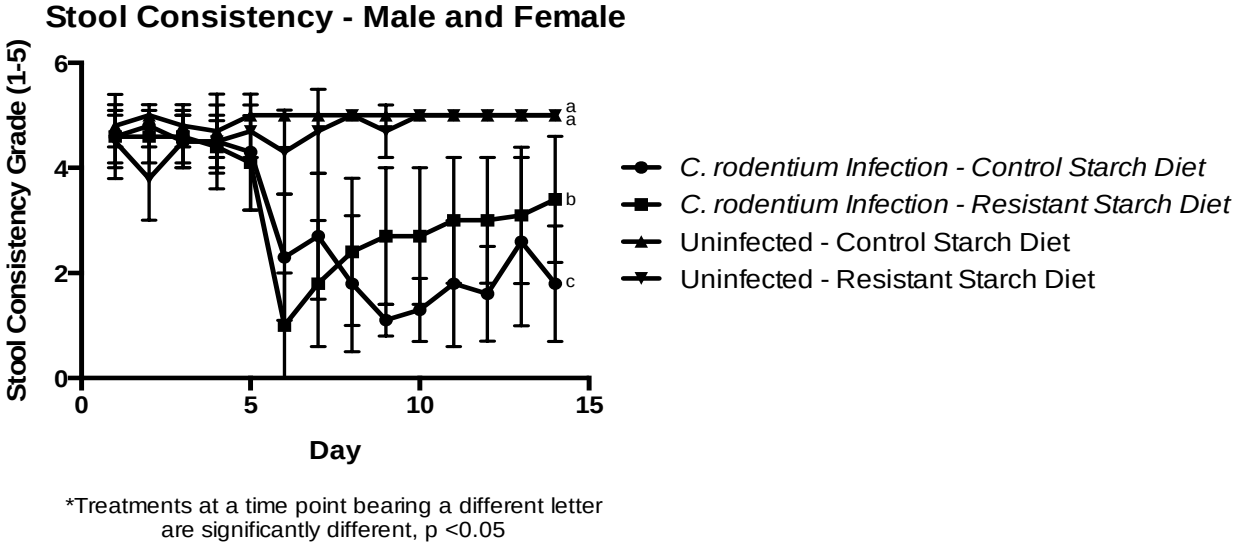
No significant difference was observed for stool consistency for day 1, 3, and 4. Uninfected mice on the RS4 diet displayed a significantly lower stool consistency than the other uninfected group. On day 5, 7, 8, 11, and 13, *C. rodentium* infected mice had significantly lower stool consistencies than the uninfected groups. However, on day 5, the stool consistency for uninfected mice on the RS4 diet was similar to those of the infected mice. The lowest stool consistency observed occurred on day 9, where the infected mice on the control diet displayed a stool consistency of 1.1 ± 0.3. The infected mice on the RS4 diet never reached a consistency as low as those on the control diet, where 1.8 ± 1.2 was the lowest stools core reached. After day 7, stool consistency for infected mice on the RS4 diet increased through day 14. On day 14, although not statistically similar to the uninfected mice, the infected mice on the RS4 diet displayed an increased in stool weight that was significantly higher than the infected mice on the control diet (Table 4, Figure 4A, 4B).

Table 4. Stool consistency (grade 1-5) for all treatments.

Infection	Starch	N	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Uninfected	Control	6	4.8 ± 0.4	5^{b}	4.8 ± 0.4	4.7 ± 0.5	5^{b}	5^{b}	5^{b}	5^{b}	5^{c}	5^{c}	5^{b}	5^{c}	5^{b}	5^{c}
Uninfected	RS4	6	4.5 ± 0.5	3.8 ± 0.8^{c}	4.5 ± 0.5	4.5 ± 0.5	4.7 ± 0.5^{ab}	4.3 ± 0.8^{c}	4.7 ± 0.8^{b}	5^{b}	4.7 ± 0.5^{c}	5^{c}	5^{b}	5^{c}	5^{b}	5^{c}
C. rod.	Control	12	4.6 ± 0.8	4.8 ± 0.4^{ab}	4.5 ± 0.5	4.5 ± 0.9	4.3 ± 1.1^{a}	2.3 ± 1.2^{a}	2.7 ± 1.2^{a}	1.8 ± 1.3^{a}	1.1 ± 0.3^{a}	1.3 ± 0.6^{a}	1.8 ± 1.2^{a}	1.6 ± 0.9^{a}	2.6 ± 1.6^{a}	1.8 ± 1.1^{a}
C. rod.	RS4	11	4.6 ± 0.5	4.6 ± 0.5^{ac}	4.6 ± 0.5	4.4 ± 0.5	4.1 ± 0.9^{a}	3.1 ± 1^{ac}	1.8 ± 1.2^{a}	2.4 ± 1.4^{a}	2.7 ± 1.3^{b}	2.7 ± 1.3^{b}	3 ± 1.2^{a}	3 ± 1.2^{b}	3.1 ± 1.3^{a}	3.4 ± 1.2^{b}

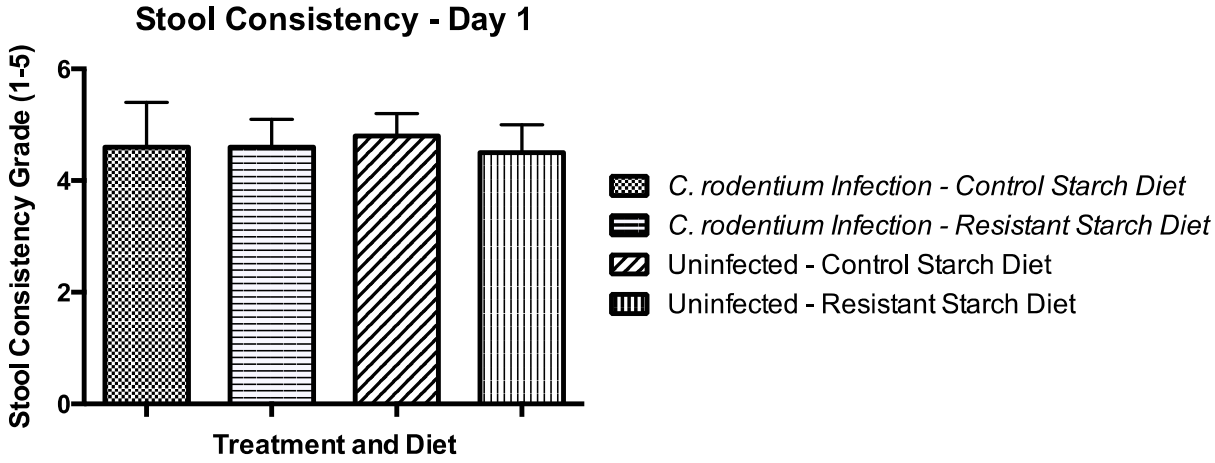
* Treatments at a time point bearing a different letter are significantly different, p <0.05; *Citrobacter rodentium* is abbreviated as *C. rod.*

Figure 4A. Stool Consistency (grade 1-5) for all treatments.

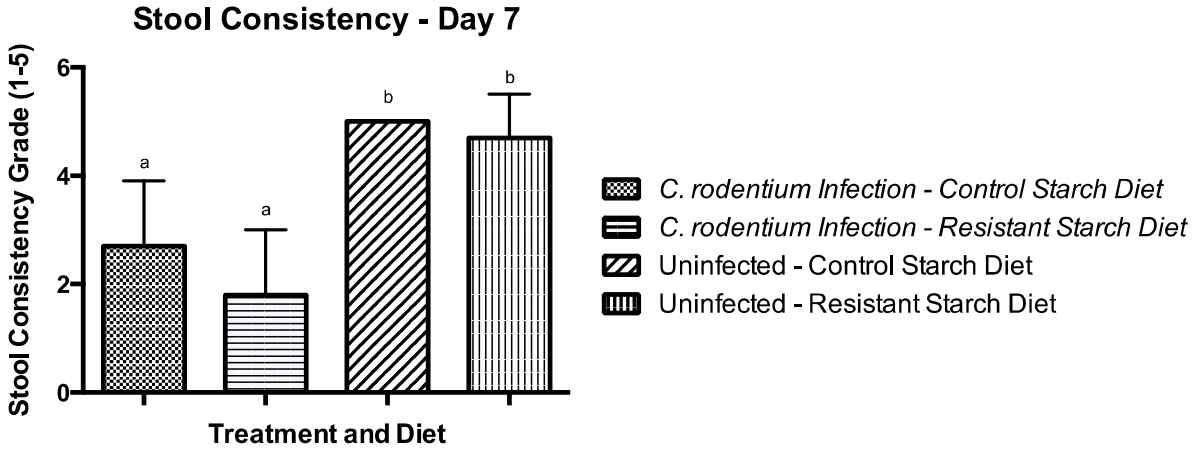


*Treatments at a time point bearing a different letter are significantly different, p <0.05

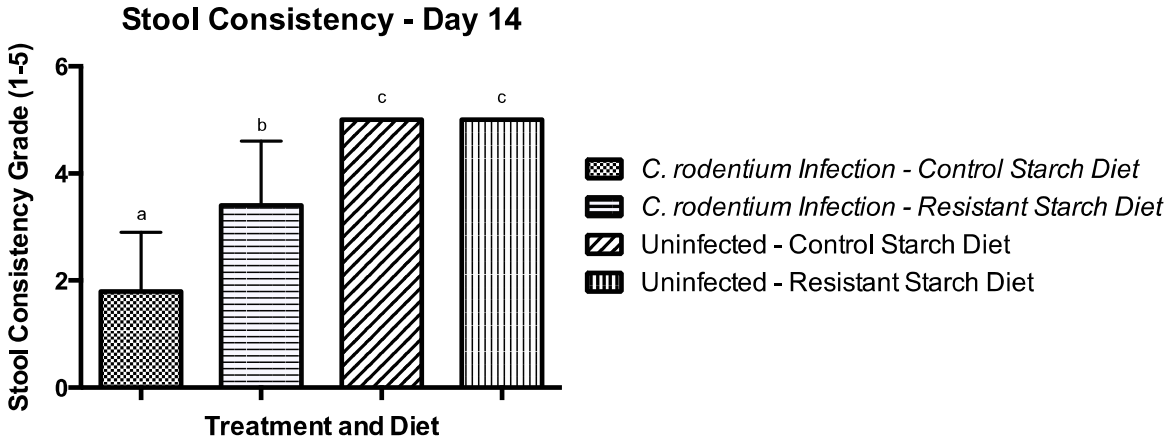
Figure 4B. Stool consistency grading for Day 1, 7, and 14.



* Treatments at a time point bearing a different letter are significantly different, p <0.05



* Treatments at a time point bearing a different letter are significantly different, p <0.05



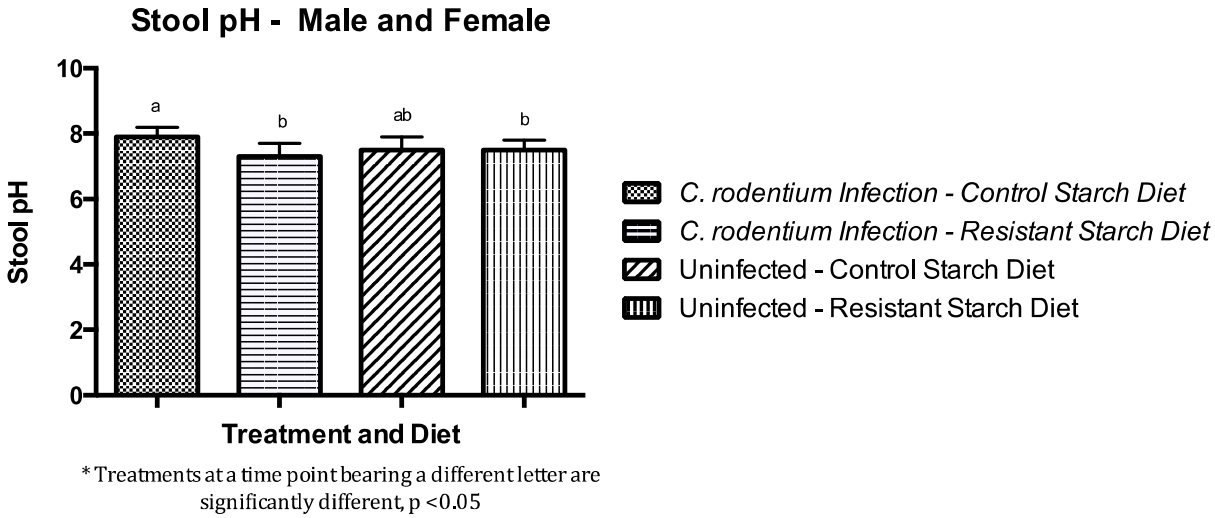
* Treatments at a time point bearing a different letter are significantly different, p <0.05

Stool Weight, Stool pH, Stool Fat, and Water and Fat Content of Stool

No significant differences were observed in stool weight for the last two days of the experiment. The ratio of stool weight to food intake also showed no significant differences. No significant differences were observed for water content of stool, which was recorded as a measure of diarrhea. No significant differences were observed for fat content of the stool (Table 5).

For stool pH, mice fed the RS4 diet had statistically similar results for pH. The values for pH for RS4 fed mice were significantly lower than the *C. rodentium* infected mice on the control diet. However, the pH value for the RS4 fed mice was not significantly different from the uninfected mice fed the control diet (Table 5, Figure 5).

Figure 5. Stool pH by treatment and diet.



* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$

Table 5. Stool weight, stool weight to food intake ratio, stool pH, water content of stool, and fat content of stool results.

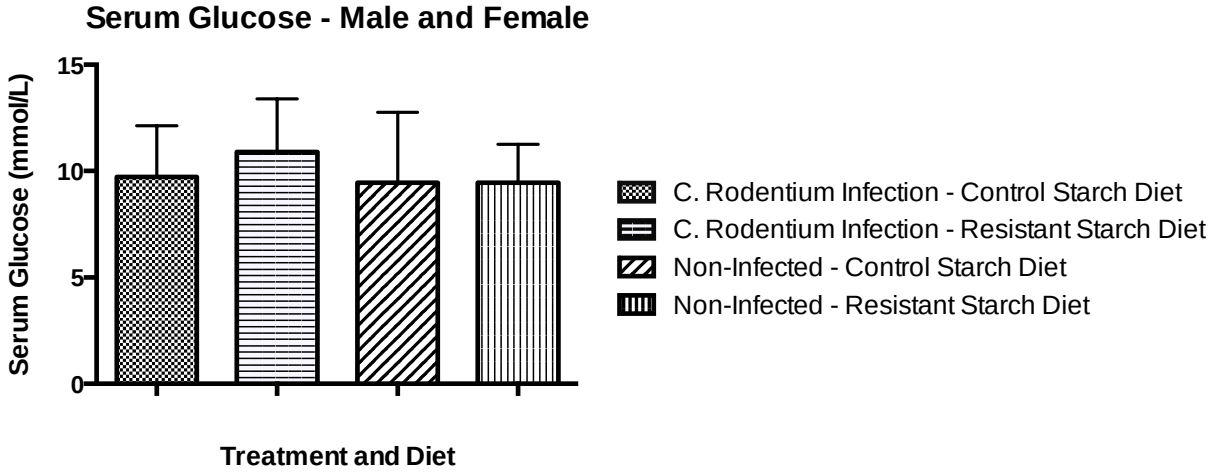
Infection	Starch	N	Day 12 Stool Weight (g)	Day 13 Stool Weight (g)	Day 12-13 Stool Weight/ Food Intake	Day 13-14 Stool Weight/ Food Intake	Stool pH	Water Content of Stool	Stool Fat per gram Stool
Uninfected	Control	6	0.25 ± 0.15	0.48 ± 0.52	0.039 ± 0.028	0.07 ± 0.086	7.5 ± 0.4^{ab}	0.6	0.14 ± 0.04
Uninfected	RS4	6	0.3 ± 0.17	0.15 ± 0.08	0.061 ± 0.04	0.06 ± 0.095	7.5 ± 0.3^{b}	0.6 ± 0.3	0.29 ± 0.34
C. rod.	Control	12	0.17 ± 0.16	0.22 ± 0.13	0.035 ± 0.043	0.032 ± 0.022	7.9 ± 0.3^{a}	0.7 ± 0.1	0.18 ± 0.17
C. rod.	RS4	11	0.3 ± 0.13	0.57 ± 0.84	0.038 ± 0.015	0.075 ± 0.088	7.3 ± 0.4^{b}	1.3 ± 2.5	0.14 ± 0.06
* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$; *Citrobacter rodentium* is abbreviated as *C. rod.*									

Serum Glucose, Insulin, and Lipids

No significant differences were observed for fasting serum glucose, insulin, total cholesterol, and non-HDL Cholesterol (Table 6, Figure 6A, 6B, 6C, 6E). Infected mice on the control starch diet displayed a lower fasting HDL cholesterol concentration, at 75.9 ± 9.1 mmol/L, than the respective mice on the control starch diet, which had serum HDL concentrations of 109.2 ± 11.8 mmol/L. Serum HDL concentrations in resistant starch fed mice did not show any significant differences from other treatments (Table 6, Figure 6D). Infected mice on the control starch diet displayed significantly lower triglyceride concentrations than the infected mice on the RS4 diet. This difference was not seen between diets in the uninfected mice (Table 6, Figure 6F). Table 6 summarizes the serum concentrations of glucose, insulin, and lipids.

Figure 6. Fasting serum A. Glucose (mmol/L), B. Insulin (pmol/L), C. Total Cholesterol (mmol/L), D. HDL-Cholesterol (mmol/L), E. Non-HDL Cholesterol (mmol/L), and F. Triglycerides (mmol/L)

A.



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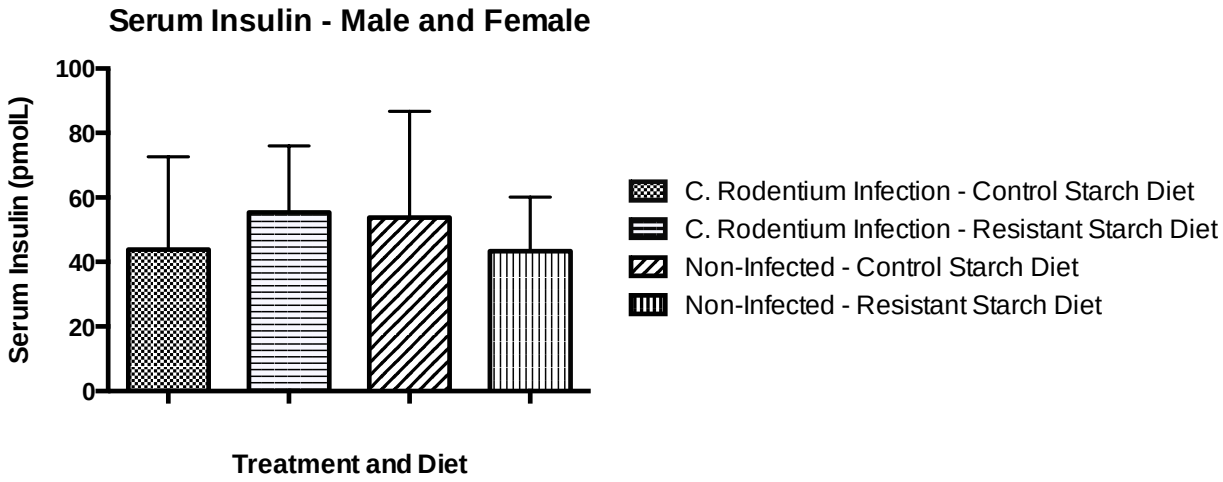

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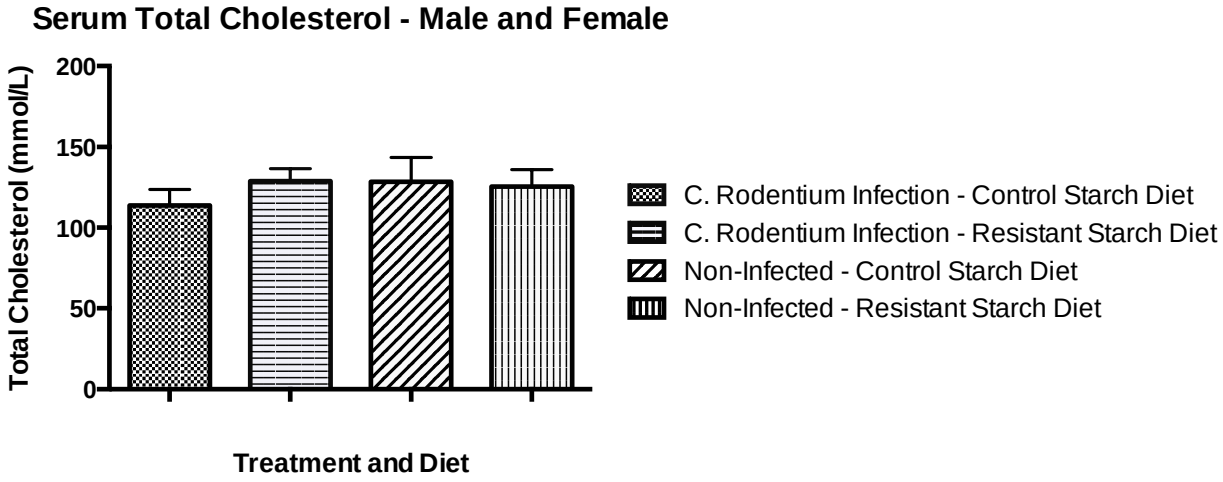

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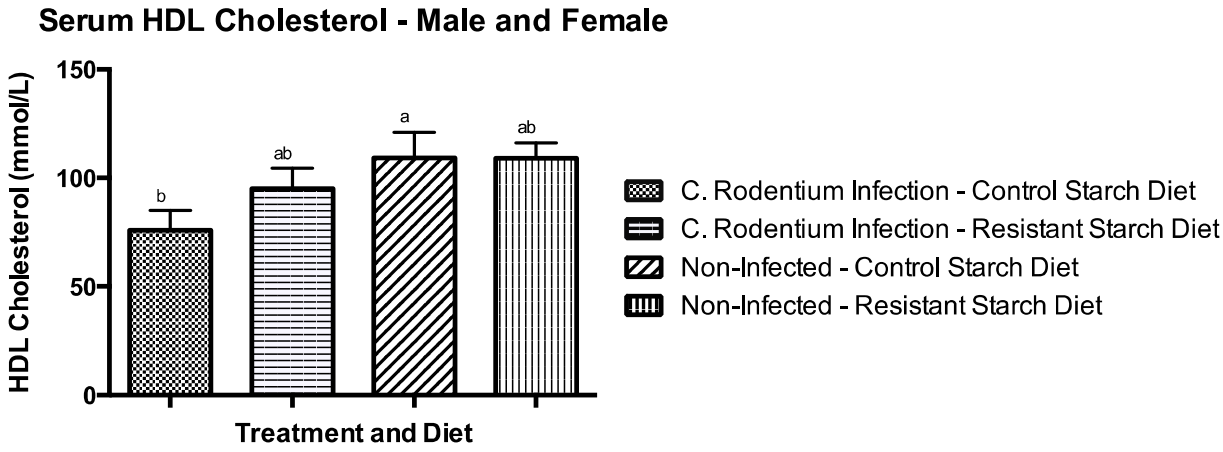
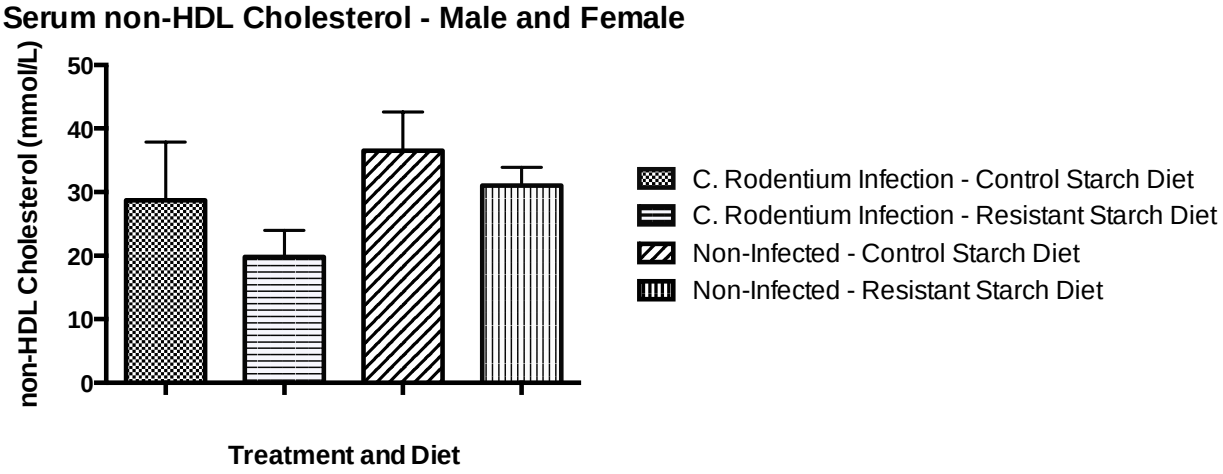


* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$

E.



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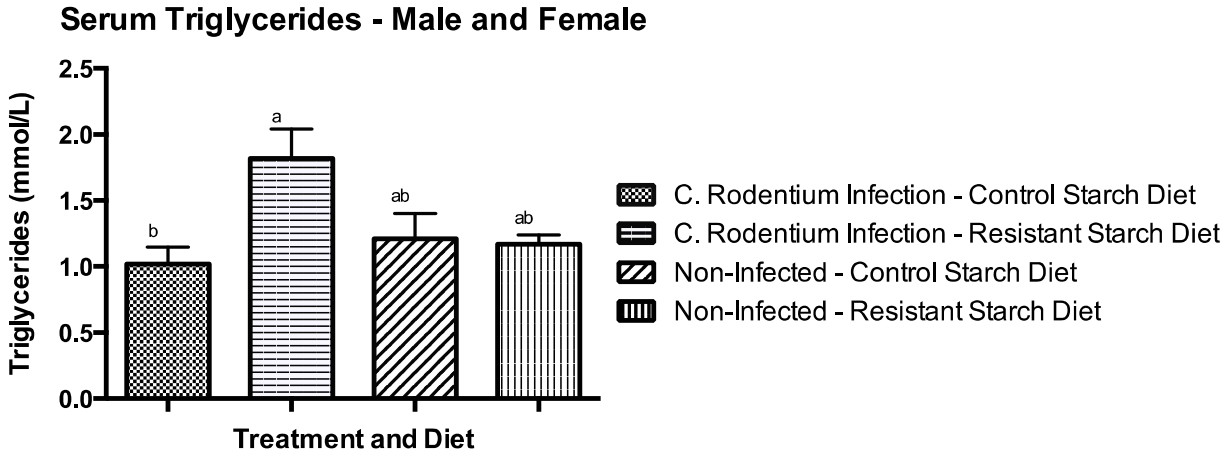


* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$

Table. 6 Fasting serum glucose, insulin, and lipid by treatment.

Infection	Starch	N	Glucose (mmol/L)	Insulin (pmol/L)	Total Cholesterol (mmol/L)	HDL Cholesterol (mmol/L)	Non-HDL Cholesterol (mmol/L)	Triglycerides (mmol/L)
Uninfected	Control	6	9.46 ± 3.3	53.7 ± 33	138.5 ± 15.1	109.2 ± 11.8[a]	36.5 ± 6.1	1.21 ± 0.19[ab]
Uninfected	RS4	6	9.46 ± 1.8	43.4 ± 16.7	125.6 ± 10.5	108.9 ± 7.1[ab]	31.0 ± 2.9	1.17 ± 0.07[ab]
C. rod.	Control	12	9.72 ± 2.4	43.8 ± 28.8	113.8 ± 10.0	75.9 ± 9.1[b]	28.7 ± 9.2	1.02 ± 0.13[b]
C. rod.	RS4	11	10.9 ± 2.5	55.4 ± 20.6	128.7 ± 7.8	95.1 ± 9.4[ab]	19.8 ± 4.2	1.82 ± 0.22[a]
* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$; *Citrobacter rodentium* is abbreviated as *C. rod.*								

Histopathology

No significant differences between treatments and diets were observed for stromal collapse (Table 7A, Figure 7E). Mucosal height, epithelial injury/ulceration, gland hyperplasia, and goblet cell change was significantly increased in *C. rodentium* infected mice (Table 7A, Figure 7A, 7B, 7F, 7G). No significant differences were observed between diet treatments for those parameters. Edema was significantly lower in *C. rodentium* infected mice than in uninfected mice (Table 7A, Figure 7D). Inflammation score was significantly higher in infected mice on the control starch diet compared to the uninfected mice on the respective diet. However, there was no statistical difference between inflammation score between both RS4 fed groups (Table 7A, Figure 7C). When statistical analysis was completed at significance level of 0.10, infected mice fed the RS4 diet displayed significant improvements in ulceration/epithelial injury, gland hyperplasia, and goblet cell density compared to the infected mice fed the control diet; this, was significantly lower than the scores for these parameters for uninfected mice (Table 7B).

When separated by gender, no significant difference was observed for mucosal height or stoma collapse for both genders (Figure 7b, 7c). *C. rodentium* males fed the control diet displayed a significantly higher ulceration, gland hyperplasia, inflammation, and goblet cell change compared to the control starch fed and resistant starch fed uninfected mice. *C. rodentium* infected males fed the resistant starch diet displayed similar results, however for ulceration, the group exhibited a significantly lowered score compared to the *C. rodentium* infected control fed group. Uninfected male mice on the resistant starch diet scored significantly higher for edema compared to all other treatments. For females, *C. rodentium* mice displayed significantly higher goblet cell

change and gland hyperplasia scores compared to uninfected mice. For ulceration, uninfected mice scored significantly lower compared to the infected mice, however, there was no significant difference in score between the uninfected mice fed the control diet and the infected mice fed the resistant starch diet. For edema, infected mice exhibited significantly lower scores compared to the uninfected mice.

Table 7A. Histopathology results for all treatments, significance level set at 0.05.

Infection	Starch	N	Mucosal Height	Ulceration/ epithelial injury	Inflammation score	Edema	Stromal Collapse	Gland Hyperplasia	Goblet Cell Change
Uninfected	Control	6	4.67 ± 0.52^{b}	0.2 ± 0.4^{b}	2 ± 0.6^{b}	0.8 ± 0.8^{b}	0	0.3 ± 0.5^{b}	0^{b}
Uninfected	RS4	6	4.83 ± 0.41^{b}	0^{b}	2.3 ± 0.8^{bc}	0.8 ± 0.4^{b}	0	0.7 ± 0.5^{b}	0^{b}
C. rod.	Control	12	6.45 ± 0.93^{a}	2.4 ± 0.8^{a}	3.8 ± 0.4^{a}	0^{a}	0.27 ± 0.47	3.4 ± 0.8^{a}	2.8 ± 0.6^{a}
C. rod.	RS4	11	6.27 ± 1.35^{a}	1.4 ± 1.3^{a}	3.3 ± 0.6^{ac}	0^{a}	0.09 ± 0.3	2.5 ± 0.8^{a}	1.4 ± 1.3^{a}
* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$; *Citrobacter rodentium* is abbreviated as *C. rod.*									

Table 7B. Histopathology results for all treatments, significance level set at 0.10.

Infection	Starch	N	Mucosal Height	Ulceration/ epithelial injury	Inflammation score	Edema	Stromal Collapse	Gland Hyperplasia	Goblet Cell Change
Uninfected	Control	6	4.67 ± 0.52^{b}	0.2 ± 0.4^{c}	2 ± 0.6^{b}	0.8 ± 0.8^{b}	0	0.3 ± 0.5^{c}	0^{c}
Uninfected	RS4	6	4.83 ± 0.41^{b}	0^{c}	2.3 ± 0.8^{b}	0.8 ± 0.4^{b}	0	0.7 ± 0.5^{c}	0^{c}
C. Rod.	Control	12	6.45 ± 0.93^{a}	2.4 ± 0.8^{a}	3.8 ± 0.4^{a}	0^{a}	0.27 ± 0.47	3.4 ± 0.8^{a}	2.8 ± 0.6^{a}
C. Rod.	RS4	11	6.27 ± 1.35^{a}	1.4 ± 1.3^{b}	3.3 ± 0.6^{a}	0^{a}	0.09 ± 0.3	2.5 ± 0.8^{b}	1.4 ± 1.3^{b}
* Treatments at a time point bearing a different letter are significantly different, $p < 0.10$; *Citrobacter rodentium* is abbreviated as *C. rod.*									

Table 7C. Histopathology results for males, all treatments.

Infection	Starch	N	Mucosal Height	Ulceration/ epithelial injury	Inflammation score	Edema	Stromal Collapse	Gland Hyperplasia	Goblet Cell Change
Uninfected	Control	3	4.67 ± 0.58	0^{a}	1.7 ± 0.6^{a}	0.3 ± 0.6^{a}	0	0.3 ± 0.6^{a}	0^{a}
Uninfected	RS4	3	4.67 ± 0.58	0^{a}	1.7 ± 0.6^{a}	1.0 ± 0.0^{b}	0	0.3 ± 0.6^{a}	0^{a}
C. Rod.	Control	5	6.20 ± 0.40	2.3 ± 1.0^{b}	3.8 ± 0.5^{b}	0^{a}	0.40 ± 0.55	3.2 ± 0.9^{b}	2.6 ± 0.6^{b}
C. Rod.	RS4	5	6.00 ± 1.00	0.4 ± 0.5^{a}	3.0 ± 0.7^{b}	0^{a}	0	2.0 ± 0.7^{b}	0.4 ± 0.5^{a}

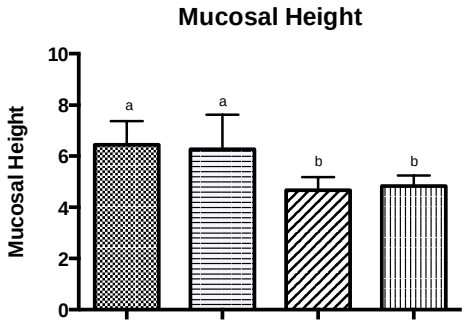
* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$; *Citrobacter rodentium* is abbreviated as *C. rod.*

Table 7D. Histopathology results for females, all treatments.

Infection	Starch	N	Mucosal Height	Ulceration/ epithelial injury	Inflammation score	Edema	Stromal Collapse	Gland Hyperplasia	Goblet Cell Change
Uninfected	Control	3	4.67 ± 0.58	$0.3 \pm 0.8a^{c}$	2.3 ± 0.6^{a}	1.3 ± 0.6^{a}	0	0.3 ± 0.6^{a}	0^{a}
Uninfected	RS4	3	5.00 ± 0.00	0^{a}	3.0 ± 0.0^{ab}	0.7 ± 0.6^{a}	0	1.0 ± 0.0^{a}	0^{a}
C. Rod.	Control	6	6.67 ± 1.21	2.5 ± 0.8^{b}	3.8 ± 0.4^{b}	0^{b}	0.17 ± 0.41	3.3 ± 0.8^{b}	3.0 ± 0.6^{b}
C. Rod.	RS4	6	6.50 ± 1.38	2.2 ± 1.2^{bc}	3.5 ± 0.6^{b}	0^{b}	0.17 ± 0.41	3.0 ± 0.6^{b}	2.2 ± 1.2^{b}

* Treatments at a time point bearing a different letter are significantly different, $p < 0.05$; *Citrobacter rodentium* is abbreviated as *C. rod.*

Figure 7a. Histopathology results.



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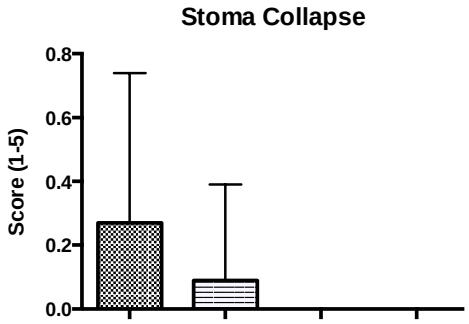

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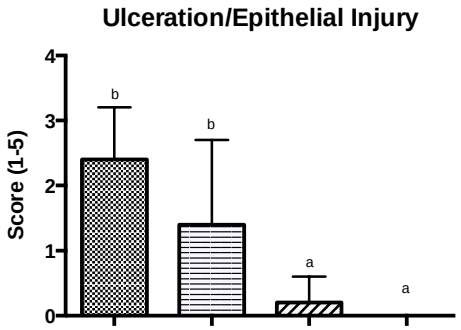

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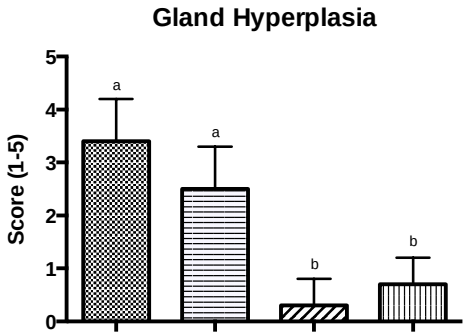

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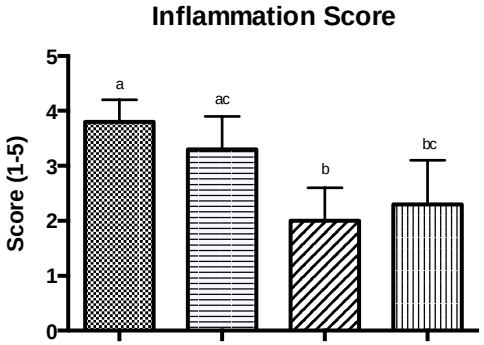

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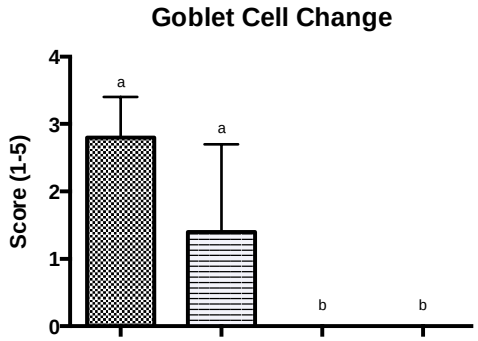

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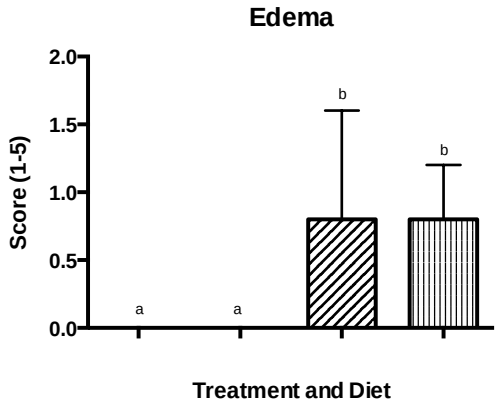

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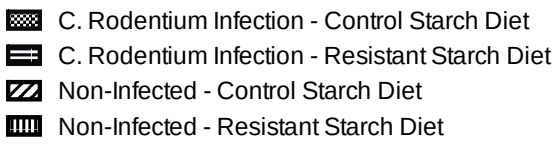


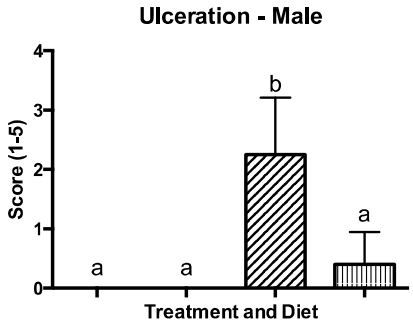
Figure 7b. Male Histopathology.

* Treatments at a time point bearing a different letter are significantly different, p <0.05

A.

* Treatments at a time point bearing a different letter are significantly different, p <0.05

E.



* Treatments at a time point bearing a different letter are significantly different, p <0.05

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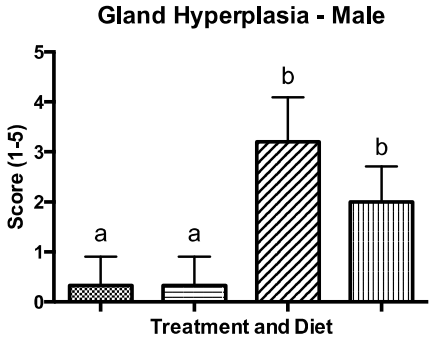
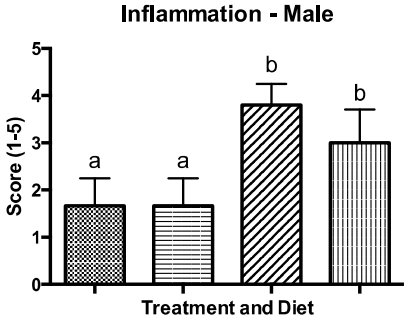


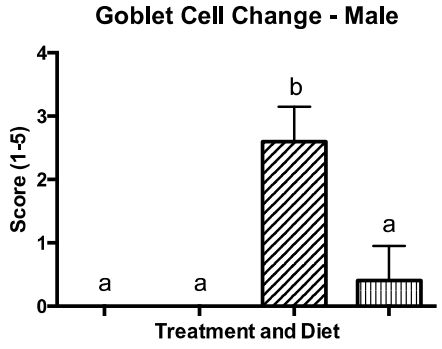
* Treatments at a time point bearing a different letter are significantly different, p <0.05

F.



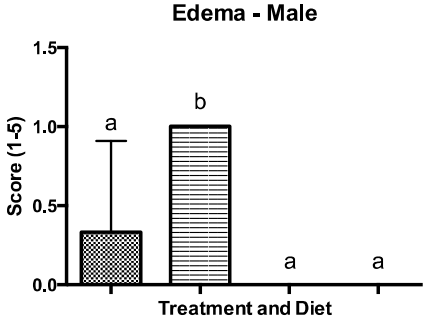
* Treatments at a time point bearing a different letter are significantly different, p <0.05

C.



* Treatments at a time point bearing a different letter are significantly different, p <0.05

G.



* Treatments at a time point bearing a different letter are significantly different, p <0.05

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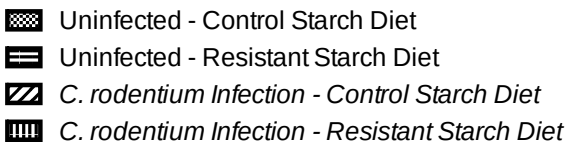
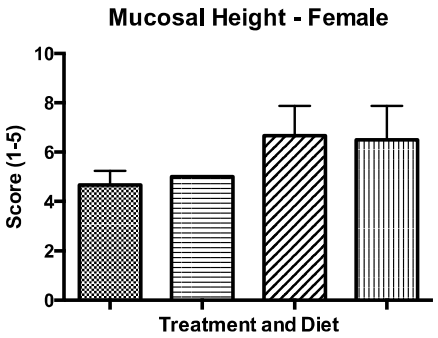
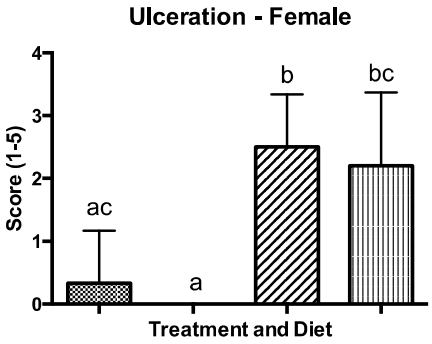


Figure 7c. Female Histopathology.



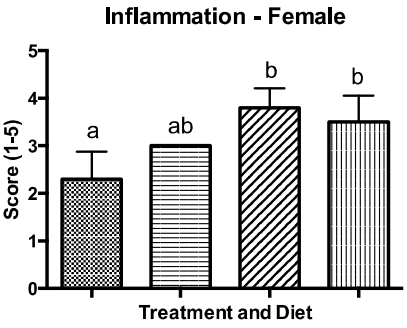
* Treatments at a time point bearing a different letter are significantly different, p <0.05

A.



* Treatments at a time point bearing a different letter are significantly different, p <0.05

B.

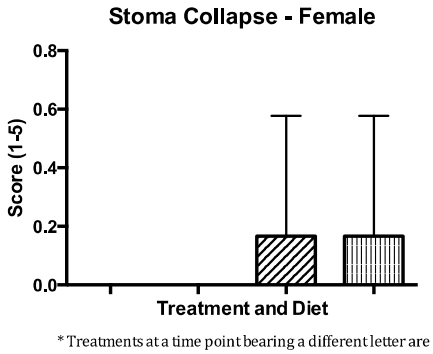


* Treatments at a time point bearing a different letter are significantly different, p <0.05

C.

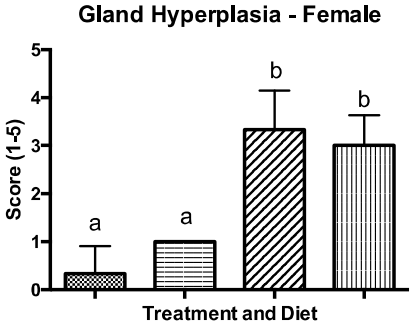
* Treatments at a time point bearing a different letter are significantly different, p <0.05

D.



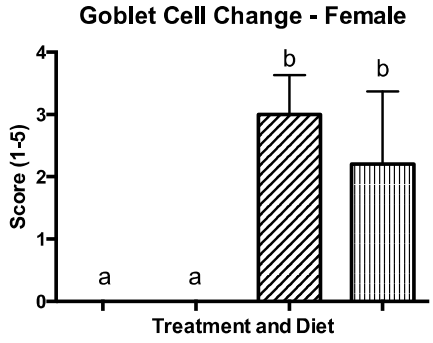
* Treatments at a time point bearing a different letter are significantly different, p <0.05

E.



* Treatments at a time point bearing a different letter are significantly different, p <0.05

F.



* Treatments at a time point bearing a different letter are significantly different, p <0.05

G.

Uninfected - Control Starch Diet
Uninfected - Resistant Starch Diet
C. rodentium Infection - Control Starch Diet
C. rodentium Infection - Resistant Starch Diet

DISCUSSION

Body Weight Changes and Food Intake

In the uninfected mice, we expected to see a lowered food intake in the mice fed the RS4 diet compared to the mice fed the control diet. Brites et al (2011) fed 36 male Wistar rats four diets consisting of wheat bread, RS-wheat bread, maize bread, or RS-maize bread for 16 days. Rats fed RS-wheat bread displayed significantly lowered food intake, stool pH, and serum glucose. RS-maize bread fed rats exhibited reduced body weight gain, stool pH, and total cholesterol level. The results concluded that RS supplementation in diets significantly reduce food intake (Brites, Trigo et al. 2011). Aziz et al (2009) performed a three-week study consisting of 46 male Sprague-Dawley rats fed a non-energy restricted high amylose diet or a non-energy restricted high amylopectin diet. The high amylose diet fed rats displayed a significantly lower energy intake and weight gain. Aziz et al also concluded that a RS diet reduced energy intake in obese rats (Aziz, Kenney et al. 2009). Bodinham et al (2010) also completed a short-term study with twenty young adult males on the effects of a dose of 48g RS on food intake for 24 hours post meal, and experienced a similar result; there was reduced food intake during the 24 hours post RS meal (Bodinham, Frost et al. 2010). This correlates with the uninfected mice, on day 13, experienced a significantly lowered food intake than the mice on the control diet.

We did not see this effect in the *C. rodentium* infected mice. Food intake was statistically similar to the intake observed in the uninfected mice fed the control diet

on days 1, 6, 11, 12, and 13. We also did not see the effect of lowered food intake in uninfected mice until the last day of the study.

Due to our hypothesis of lowered food intake, we also expected to see lower body weights in mice fed the RS diet. This hypothesis was not observed in either group of RS4 fed mice. This result could be attributed to the lack of reduced food intake in mice fed the RS4 diet, as we did not see a significantly lowered food intake until the final day of the study.

We expected that the *C. rodentium* treatment would cause severe weight loss in our mice. Weight loss in the *C. rodentium* infected mice is potentially due to the disruption of intestinal barrier function, leading to malabsorption of nutrients, ultimately resulting in weight loss (Hodges and Gill 2010). Other potential reasons for weight loss in the *C. rodentium* model include loss of nutrients in stool or increased energy expenditure. Rigaud et al (1994) analyzed weight loss in 30 Crohn's disease patients, which is an inflammatory bowel disease with symptoms similar to those of the *C. rodentium* model. The study concluded that weight loss in this type of disease may be due to a decrease in food intake (Rigaud, Angel et al. 1994). In the infected mice, we observed an initial decrease in body weight for the mice fed the control starch diet, starting at day 6. This continued through day 13, where weight loss was significantly lower compared to the uninfected mice fed the control diet. This was not observed in the infected mice fed the RS4 diet. Body weights were maintained throughout the study, and were not significantly altered from the uninfected mice. When separated by sex, males showed similar results, where the infected mice fed the control starch diet had significant weight loss

compared to all other treatments. We did not, however, see a significant weight loss in the infected mice fed the control diet. Females showed similar body weight changes throughout the study for all treatments. This can be attributed to the lack of a significant difference in food intake for female mice, in all treatments.

The results that body weight and food intake did not significantly change in RS4 fed mice infected with *C. rodentium* suggests that a resistant starch supplemented diet could potentially protect from the weight loss associated with colitis, or other inflammatory bowel diseases.

Stool Consistency

We hypothesized that mice inoculated with *C. rodentium* would have a large decrease in stool consistency, but those on the RS4 diet would have a less severe diarrheal response than those on the control diet. The decline in stool consistency for those infected started at about day 6 and 7. After the initial decrease, the infected mice on the RS4 diet experienced a steady increase in stool consistency (Figure 4A) By the final day, the infected mice on the RS4 diet had a significantly higher stool consistency (~3.5) than the infected mice on the control diet (~2), confirming our hypothesis (Figure 4B). The infected mice on the RS4 diet, however, did still have a significantly lower stool consistency than non-infected mice. This suggests that the RS mitigated diarrhea, but did not completely improve upon stool consistency.

RS can improve stool consistency in a number of ways. It has a high water holding capacity, allowing it to isolate water from the liquid diarrhea. Absorption of the water in diarrhea by RS increases stool bulk, which would contribute to our

higher stool consistency observed in *C. rodentium* infected mice on the RS4 diet (Bosaeus 2004).

Production of short chain fatty acids (SCFAs) is another contributing factor for the decrease in diarrhea due to consumption of RS. Through fermentation in the large intestine, short chain fatty acids are produced, which decrease the pH in the gut. This decrease in pH, along with the SCFAs can stimulate water and sodium absorption in the large intestine. Increased water absorption increases stool consistency, and ultimately decreases the diarrhea associated with the *C. rodentium* infection (Soral-Smietana and Wronkowska 2004). In our study, however, fecal SCFAs were not measured, so this correlation could not be confirmed.

Ramakrishna et al (2000) discovered that the addition of RS in oral rehydration therapy reduces fluid loss in stool and shortens the duration of diarrhea in adults and children. 48 adolescents were randomly assigned to glucose based oral rehydration therapy (n=16), glucose oral rehydration therapy with 50 g/L rice flour (n=16), or glucose oral rehydration therapy with 50 g/L high amylose maize starch (n=16, and stool weight and transit time to first stool were measured every 12 hours for 48 total hours (Ramakrishna, Venkataraman et al. 2000). In another study, Ramakrishna et al (2008) subjected 50 adult males to hypo-osmolar oral rehydration solution with or without 50g/L of high amylose maize starch substituted for glucose. Parameters were measured for 48 hours, which included consistency and weight of stool. Stool consistency was based upon the Bristol scale. Ramakrishna et al found that oral rehydration of RS reduced diarrhea diarrheal

duration, leading to the belief that RS could be used as a treatment for diarrheal diseases, including cholera (Ramakrishna, Subramanian et al. 2008).

We found that although there was a significant increase in stool consistency with *C. rodentium* infected mice fed the RS4 diet compared to the infected mice on the control diet, the stool consistency in the infected mice fed the RS4 diet was still significantly lower than the uninfected mice. The results found in this study, along with the results found in the literature, can contribute to the indication that RS can improve diarrhea, making it a useful method of treatment for diarrheal disease.

Stool pH

A significantly lowered stool pH was observed in the *C. rodentium* infected mice fed the RS4 diet compared to the infected mice fed the control diet. Lowered pH in the stool suggests a lowered gut pH, which indicates a higher level of SCFA presence. This higher level of SCFAs indicated by the stool pH can promote ion uptake and water uptake, therefore decreasing diarrhea. The observation that the RS4 diet decreased pH in the stool corresponded with the increased stool consistency observed in RS4 fed infected mice.

We found that in infected mice, the RS4 fed group had a significantly lower stool pH compared to those fed the control diet. These results indicate that the RS can mitigate diarrhea through a lowered gut pH, which may be attributed to SCFA production through fermentation of the RS4 in the large intestine.

Serum Glucose, Insulin and Lipids

We hypothesized that a RS4 supplemented diet would decrease insulin and glucose responses in our mice. Resistant starches are associated with a higher composition of amylose, which contributes to the indigestibility. The chain-like nature of amylose causes glucose to be released slowly, causing less glucose to be absorbed in the small intestine. The glucose response of an organism, in turn, would decrease in conjunction, due to the lowered availability of free glucose. A lowered insulin response would be observed as well, because the lessened availability of free glucose would have a lowered stimulation on insulin. The stability of glucose and insulin response due to resistant starch would lead to stability in body weight (Nugent 2005, Brites, Trigo et al. 2011). This effect of a lowered insulin and glucose response was not observed in our mice.

Lowered cholesterol levels in blood due to RS are thought to be caused by synthesis of SCFAs, which can lower cholesterol synthesis in the liver. (Vanhoof and De Schrijver 1998, Fernandez, Roy et al. 2000). The primary SCFA associated with these lowered levels of lipids is propionate. Propionate is metabolized in the liver to create acetyl-CoA, which would attenuate cholesterol and fatty acid synthesis in the liver, as well as increasing HDL cholesterol production (Soral-Smietana and Wronkowska 2004). We did not observe a lowered triglyceride concentration or total cholesterol in mice fed the RS4 supplemented diet, nor did we observe a higher HDL cholesterol concentration in the RS4 fed mice.

Histopathology

We predicted that the *C. rodentium* infection would cause severe inflammation in the lining of the colon. We hypothesized that the mice on the RS4 diet would have mitigation of the inflammatory responses, indicating protection from the resistant starch.

C. rodentium causes epithelial hyperplasia in the colon (Borenshtein, Nambiar et al. 2007). Wang et al (2006) found that in male and female Swiss-Webster mice, gland hyperplasia was at its maximum at 12 days post inoculation of *C. rodentium* (Wang, Xiang et al. 2006). In our study, we found that there was a significant increase in hyperplasia in infected mice. At $p<0.05$, there was not a significant decrease in hyperplasia score for infected mice fed the RS4 diet compared to the uninfected mice. However, when $p<0.10$, the RS4 fed infected mice displayed a significantly lower hyperplasia score compared to the control diet fed infected mice. Although not significantly similar to the hyperplasia score uninfected mice, the lowered hyperplasia score indicates a partial protective effect of the resistant starch on hyper proliferation of cells associated with inflammation.

Other histological changes associated with *Citrobacter rodentium* include the following: goblet cell loss, and ulceration (Borenshtein, Nambiar et al. 2007). Higgins et al (1999) found that by day 6 post inoculation with *C. rodentium*, female Swiss NIH and C3H mice began to experience a significant thickening of the colon (Higgins, Frankel et al. 1999). We predicted that mice infected with *C. rodentium* would exhibit these responses, but mice fed the RS4 diet would have a lessened inflammatory response. In our study, mice infected with *C. rodentium* displayed a

significantly higher score for ulceration and goblet cell loss. However, at $p<0.05$, there was no significant difference observed between the RS4 diet and control starch diet in infected mice. At $p<0.10$, scores for ulceration and goblet cell loss were significantly lowered in RS4 fed infected mice compared to control starch fed mice. This potentially shows a protective effect of the resistant starch treatment to the inflammatory effects brought on by *C. rodentium*. The inflammation score for *C. rodentium* infected mice did increase compared to uninfected mice. However, the scores for inflammation between both treatments on the RS4 diet were not significantly different. This supports the conclusion of partial protection by the resistant starch diet on the effects created by *C. rodentium*.

The lessened severity of inflammatory responses due to the RS diet could be attributed to the indigestibility of the starch, and its passage to the large intestine. The fermentation of resistant starch in the large intestine produced short chain fatty acids. An important SCFA produced is butyrate. The production of this butyrate stimulates the function of enterocytes in the gut, which use it as an energy source. This increases the cells capability of overcoming the disruption of barrier function caused by pathogens like *C. rodentium* (Jacobasch, Schmiedl et al. 1999). The ability of the RS4 to be fermented in the large intestine to a higher degree than the control starch lead to a higher SCFA production in the gut. This opens up the potential of using type-4 resistant starch as a treatment option to decrease or protect against inflammation associated with diseases like colitis.

We observed a significant improvement of inflammatory responses including gland hyperplasia, ulceration, and goblet cell loss when $p<0.10$. This improvement,

although still significantly lower than the uninfected mice, suggests a partial protection by RS4 against the inflammation associated with the *Citrobacter rodentium* model. The partial protection of the RS introduces RS4 as a protective treatment for inflammation in colitis.

Limitations

This study was a short-term study, only 14 days. Although significant findings were observed, a longer study may have revealed more benefits to the RS diet. *C. Rodentium* is a self-limiting infection, and lasts only 3-4 weeks before being cleared (Borenshtein, McBee et al. 2008). This would not allow for a longer study, due to some of the decline in inflammation and increase in stool consistency being caused by the clearance of the infection.

The correlation between SCFA production and mitigation of diarrhea was not able to be determined in this study. A further study of the mitigating effects of diarrhea due to RS consumption and its relation to fecal SCFA or SCFA production in the gut would be necessary to confirm this notion.

General Conclusions

Our results indicate that a diet supplemented with RS4 can lessen the severity of diarrhea caused by the *Citrobacter rodentium* A/E pathogen. Future research should be considered to probe the mechanism of type-4 resistant starch on gut barrier function, leading to this mitigation of diarrhea. Our results also suggest a partial protection by RS4 against inflammatory responses associated with the

Citrobacter rodentium pathogen. Additional research investigating the mechanisms of RS4 on inflammatory responses is necessary as well. The outcome of additional research could create a RS supplemented food product used as an anti-diarrheal and anti-inflammatory dietary treatment.

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